An integrative approach to understanding conflict management in a highly social vertebrate

Tommaso Francesco Ruberto

A thesis submitted in partial fulfilment of the requirements of

Liverpool John Moores University for the degree of Doctor of Philosophy

July 2023

T. F. Ruberto, 2023

List of contents

Chapter 1: Introduction

Chapter 2: Submissive signalling in subordinate daffodil cichlids

Chapter 3: Submissive behaviour is affected by territory quality in a group living cichlid fish

Chapter 4: Dominance and aggression correlate with vasotocin neuronal phenotypes in a highly social fish

Chapter 5: Arginine-vasotocin involvement in the establishment of daffodil cichlid social hierarchies

Chapter 6: Discussion

Abstract

Sociality encompasses a wide and complex array of socially relevant interactions and cognitive processes, such as approaching conspecifics, individual recognition, and discrimination, parental and alloparental care, and conflict management tactics. Such behaviours often emerge through gradual modifications in behaviour, driven by subtle changes in underlying physiological mechanisms. Recognizing the functional behavioural building blocks at the basis of these transformations is crucial for understanding the evolution of complex social behaviour and group living patterns, and gaining insights into how social animals handle conflicts. In this PhD thesis, I explored the intricacies of agonistic behaviours in the highly social cooperatively breeding daffodil cichlid fish (*Neolamprologus pulcher*) and the associated regulatory mechanisms using diverse perspectives. I performed experiments to study the role of the putative submissive signal head-up display (HUD) during the resolution of conflicts, the effect of environment in the expression of agonistic behaviours, the role of the nonapeptide hormone arginine vasotocin (AVT) on the regulation of social behaviours and in the development of dominance hierarchies. I used an integrative approach encompassing behavioural tests on daffodil cichlids social groups, territorial contest-based experiments, fluorescent immunohistochemistry, and analysis of immediate early genes associated with AVT neuronal activity. The HUDs were utilised as a submission signal by subordinate daffodil cichlids and were strongly correlated with the reduction on the frequency of aggression from the receiver. Aggression of dominant fish was affected by the environment, as well as a rank-based submissive response of the subordinates. The immunohistochemistry experiments showed that AVT is closely related to the individual status in the group social hierarchy and to the dominant individual's aggression, but not to the submission levels of the subordinates. Although there were no differences in AVT cell activity associated with the expression of immediate early genes, there was a trend of positive correlations between aggressive behaviours and AVT activated cells, which supports the possibility of future studies. Findings of this thesis contribute to a better understanding of the subtleties involved in agonistic interactions within daffodil cichlids, an

emerging model for the investigation of sociality. Finally, the questions in this thesis highlight the importance of delving deeper into the study of social behaviours, to identify and achieve a more comprehensive comprehension the underpinning mechanisms at the basis of conflict management and agonistic behaviours.

Declaration

I declare that no portion of the work referred to in the thesis has been submitted in support of an application for another degree or qualification of this or any other university or other institute of learning.

Acknowledgements

Firstly, I would like to thank my supervisors at LJMU, Dr. Adam Reddon and Dr. Will Swaney. I shared with them more than 3 years of my life, they are people of great intelligence and humanity, passionate about their research, and always ready to help. I am grateful for the invaluable advice and important scientific feedback that they provided throughout the journey of my Ph.D. project, along with the precious support during the writing of my thesis and of the papers that we published. I would also like to thank my external supervisor Dr. Hazel Nichols, who helped me with her feedback to shape my research and write this thesis. It was a privilege and an honour for me to work with all of them.

I could not have accomplished this journey without the love and support (and patience) of my partner, Claudia, especially during the years of the pandemic. We spent a lot of time away from each other, but she always believed in me, and she constantly encouraged me to finish my Ph.D., even throughout the difficult times.

A sincere thank you goes to my family: thank you, for all your love and support, and for putting your trust in me, despite thousands of kilometres of distance.

Thank you to Stefano and his wife Sabrina, I am grateful and lucky to have such an amazing couple of friends. I would also like to acknowledge and thank Natalia, Jeff, Mike and Veronica, the colleagues who I have worked with in the past few years and from who I learnt a lot. Thank you to all my friends from Italy, to the friends that I have met during my 3 years in Liverpool, and to my favourite housemates, Natalia and Matt. Lastly, I wish to thank as well LJMU for providing funding for my research.

Chapter 1: Introduction

1.1 Sociality in animals, its benefits, and its burdens

While animal sociality has been a topic of study for decades, defining different social systems is not necessarily straightforward (Krause & Ruxton, 2002), as even the so-called solitary species must interact with other conspecifics, and potentially form a social group, once in their life (Tinbergen, 1953): For instance, animals may gather for mating purposes or for taking care of their offspring, or even when a considerably large food source becomes available and attracts many individuals. Thus, the definition of “group living species” varies depending on the occurrence and the kind of expected interactions that conspecifics should exhibit between each other to create a social group, how they are coordinated and cohesive, and how consistent is their group (Alexander, 1974; Krause & Ruxton, 2002). A general definition from Wilson (2000) states that a social group is “any set of organisms, belonging to the same species, that remain together for a period of time while interacting with one another to a distinctly greater degree than with other conspecific organisms.”, although heterospecific sociality, that is, sociality involving individuals of multiple species, has been documented across various taxa and ecological contexts (Boinski & Garber, 2000; Stensland et al., 2003; Seppänen et al., 2007; Goodale et al., 2010).

According to the general consensus, solitary living is regarded as an ancestral condition, and that group living has independently evolved in several different taxa (Majolo & Huang, 2022). Many theories have been suggested on such evolution, ranging from species-specific hypotheses to more general ones that could be applied to any taxa (Sumpter, 2010). The costs and benefits of solitary versus group living for the individual fitness are usually considered as the main ecological drivers for the evolution of sociality. Such ecological drivers provide substantial advantages to individuals living in groups over solitary living and may be categorised in three major groups: (1) access to resources such as water, food, and shelter; (2) mating; and (3) anti-predatorial strategies (Majolo et al., 2008; Sumpter, 2010). Forming a social group allows animals to gain access to and/or preserve the above-mentioned resources to the detriment of solitary animals or smaller groups from the same species (Alexander, 1974; Packer & Ruttan, 1988; Krause & Ruxton, 2002). Within this framework, sociality may have developed at some point in several different species when the benefits of group living for the fitness of the individual in a group outweighed its costs (Sumpter, 2010; Majolo & Huang, 2022). Examples of such benefits are found among species of different phyla: fishes may form coordinated schools to reduce predation risks (Partridge, 1982; Moyle & Cech, 1996); living in a group increases the chances of capturing prey for several mammalian carnivore species (Macdonald, 1983); in some marine bird species, individuals living in colonies may help other conspecifics in raising their offspring (Brown, 2014).

Looking for food in an environment may be a time-consuming and potentially dangerous activity, as it exposes animals to potential predators, especially when an animal is alone. In this perspective, living in a group has the advantage of information sharing (i.e., food location and availability, area assessment in terms of suitability for the group or of the perceived risks, or nearby presence of predators) among group members (Macdonald, 1983; Packer & Ruttan, 1988). The benefits of group foraging are found among different taxa. For example, goldfish (*Carassius auratus*) and common minnows (*Phoxinus phoxinus*) may locate food sources more quickly when their group size is bigger (Magurran & Pitcher, 1983; Stenberg & Persson, 2005), and foraging in groups grants the greater mouse-tailed bats (*Rhinopoma microphyllum*) increased effectiveness in detecting and capturing prey when high-levels of group density are maintained (Gager, 2019). Further examples of cooperative prey capture are found among mammalian carnivores, such as lions (Stander, 1992), African wild dogs (Creel & Creel, 1995), and wolves (Mech & Boitani, 2003).

With respect to mating, social living animals may access a mating partner more easily than those who live alone, and at the same time they may compare more potential breeding mates and therefore more choice (Daly, 1978). In lek mating species, for example, competitive displays are used by males to communicate their strength and skills to potential mating females, because good competitive abilities in males are often correlated with genetic quality. Females assess the males' qualities and strengths through the contest, thus providing the females an opportunity for choosing the right mating partner. Examples of this particular mating system are found in a variety of taxa, including insects, birds, and mammals (Bradbury, 1981; Höglund & Alatalo, 1995).

When animals are in groups, they are more likely to spot a potential predator ("many-eyes" effect (Lima, 1995)), compared to a single individual, thereby reducing the predation risk. At the same time, this strategy consents to an individual to spend more time on different activities than anti-predatorial vigilance (Lima, 1995). Additionally, the use of alarm calls or displays for signalling a predator to other group members is found among many different social species (Zuberbühler et al., 1999). Other strategies may imply the use of "sentinel" individuals, as for example among meerkats (*Suricata suricatta*), that take turn in spotting potential predators while other group members are occupied in different activities, like resting or foraging (Hollén et al., 2008). On the other hand, living in a group also facilitates individual animals to avoid predator attacks, as once a predator has been spotted, the group can either flee from it or attempt to drive it away through "mobbing" (Curio et al., 1978; Caro, 2005). Aside from serving as a deterrent to predators, engaging in mobbing behaviour, a common behaviour in meerkats (Graw & Manser, 2007) and birds (Cunha et al., 2017), may also offer valuable information for assessing the risk of predation. Likewise, through the "dilution effect", the larger the group of prey is the more challenging it gets for a predator to focus on a single target, and at the same time the less likely is for a single prey animal to get caught by the predator (Lima, 1995). For example, common redshanks (*Tringa totanus*) that are part of larger flocks have lower chance of being predated by raptor birds than those living in smaller flocks (Cresswell, 1994).

On the other hand, sociality can be potentially detrimental within group members due to an increased competition for resources, depending on their availability and distribution (Lorenz, 1966; King, 1973; Krause & Ruxton, 2002), but social animals may endure costs even when they are not monopolising and aggressively excluding other group members from that resource. Larger foraging groups animals may incur in lower quantities of potentially available food per capita (even considering inter-individual differences in feeding efficiency, animal size, etc.). For instance, in banded mongooses (*Mungos mungo*), subordinate individuals breed regularly, and dominant females that suffer from such reproductive costs may respond to the increased reproductive competition by evicting subordinates (Cant et al., 2010)

The benefits versus costs for an animal living in a group in relation to predatorial risk are defined by the interplay between the chance of the group of being detected risk by a predator and the probability of that animal to be predated. For example, although the predation risk for each pupa of stream-dwelling trichopteran (*Rhyacophila vao*) is still lower than for those pupae that live in smaller groups, larger groups are more prone than smaller groups to be attacked by planarian predator (*Polycelis coronata*) (Wrona & Dixon, 1991). Additionally, predators usually aim for individuals who are weak, sick, slower, and/or those who are at the edges of the group, so the members of group may not be all equally likely to be predated (Curio, 1976). For example, the bluegill sunfish (*Lepomis macrochirus*) preferentially focus their attacks on prey who are not well-coordinated with the group's movements (Gross & MacMillan, 1981).

Living in a group may also increase the transmission of parasites and diseases (Alexander, 1974; Schmid-Hempel, 2017). Social animals often interact among each other: as such, they may be in close proximity with potentially sick individuals and therefore increase the risk of disease/parasite transmission compared to solitary living animals. For example, in the bobwhite quail (*Colinus virginianus*), a ground-dwelling bird species, the flock size and levels of infection by parasitic helminths are positively correlated (Shea et al., 2021). Furthermore, in the European bee-eaters (*Merops apiaster*), the amount of parasite infections decreases when inter-nest distances are higher (Hoi et al., 1998). With high levels of parasitic infections, the ecological costs for the animals, in terms of slow growth, high mortality rates, and reduced reproduction, are significantly increased, however, how and to which extent the risk of parasite/disease infection hinders group size and sociality is still unclear.

Finally, in social animals, intraspecific competition may be frequent and have substantial fitness costs (Hardy & Briffa, 2013). Such competition implies interactions that may range from agonistic displays from a distance to physical contact, and the winner of the competition may have either the priority or the exclusive access to the disputed resource (Hardy & Briffa, 2013; Huntingford & Turner, 2013). However, agonistic interactions are costly, in terms of resources and time spent performing such interactions, and physical contacts may result in severe injuries, or even death, for either the loser or both the winner and the loser of the interaction (Hardy & Briffa, 2013; Huntingford & Turner, 2013). Hence, in social species, individuals must be able to cope with these dynamics to establish stable and thriving social groups (Aureli & de Waal, 2000).

1.2 How to cope with conflicts in social species

Conflict management behaviours encompass a complex interplay between dominance hierarchies and other behavioural adaptations, such as aggression, submission, and cooperation (Aureli & de Waal, 2000), and examples are found across different species of mammals (Aureli & de Waal, 2000; Flack & De Waal, 2004), birds (Clayton & Emery, 2007; Silk, 2007) and fishes (Bshary et al., 2002). Animals living in groups are expected to show some degree of within-group competition for resources (de Waal, 1986; Aureli et al., 2002; Silk, 2007), and some of these can result in prolonged and agonistic interactions (Earley & Dugatkin, 2010). Agonistic interactions among group members may be costly for the animals (Kutsukake & Clutton-Brock, 2008), and if they persist over time, the costs of such interactions may offset the benefits of grouping (Aureli et al., 2002). Minimizing the costs of such interactions is a mutual concern for the individuals engaged in a contest (Maynard Smith & Price, 1973; Maynard Smith & Harper, 2003). In this framework, chemical (Frommen, 2020) and visual cues (Maynard Smith, 1982), displays (Garamszegi et al., 2006), or vocalizations (Burgdorf et al., 2008) may also be used in “ritualized” combats, in which two opponents make minimal physical contact or target non-vulnerable body parts rather than using damage inducing attacks during a fight, as a result of this mutual interest between challenging individuals (Geist, 1966). Therefore, even if intrinsically competitive, agonistic behaviours encompass elements of cooperation between contestants (Hurd, 1997). In fact, animals usually engage in all-out fight only when the benefits of a contest victory are so high and the future fitness prospects of the loss are extremely low (Enquist & Leimar, 1990).

There are three possible outcomes for an agonistic interaction: (1) one individual is either incapacitated or killed by other contestant; (2) the loser of the contest conceeds the resource and its physical location to the winner by fleeing; and (3), the loser performs a submissive signal towards the winner, yielding the resource but being allowed to stay within the same location of the above-mentioned resource. Submission signals may be produced by a submissive individual either after fighting, to cease the interaction, or prior to any aggressive escalation, pre-emptively de-escalating the contest before it commences (Kutsukake & Clutton-Brock, 2006; Kutsukake & Clutton-Brock, 2008). As such, submissive signals benefit the signalling animal by preventing or ending a costly contest, and at the same time avoid any further aggression. The individual receiving such signal indirectly gains an advantage by reducing the energy and time needed to either start or continue an aggressive interaction, and simultaneously avoiding the chances for another contest. Moreover, in some social species the submissive signals gain an increased utility, as for example when individuals are unable to flee from a conflict because of their necessity to remain within a shared group territory (Buston, 2003; Heg et al., 2004).

Submission signals are a widespread way to end contests: for example, in veiled chameleons (*Chamaeleo calyptratus*), territorial fights end when one individual suddenly darkens their body (Ligon, 2014). Such coloration change leads to a rapid decrease in aggression by the other contestant, and the levels of aggression received are indeed tied to the probability that the recipient will darken their body (Ligon, 2014). In a similar fashion, salmonid fishes (*Salmo* spp.) signal submission towards an opponent they concede to by darkening their body and eye colouration (Keenleyside & Yamamoto, 1962; O'Connor et al., 1999; Hoglund et al., 2000; O'Connor et al., 2000; Suter & Huntingford, 2002). This darkening inhibits aggression in the receiver, resulting in a precipitous decrease in attack intensity (O'Connor et al., 1999). Submission often involves a behavioural response: for example, during contests between crayfish males (*Procambarus clarkii*) when one individual yields to a competitor by displaying a typical mating posture of a female, these pairs show fewer total aggressive behaviours, a lower level of energy spent on fighting in both individuals, and an increased chance of survival for the yielding individual, compared to pairs in which the loser does not display any submissive signal (Issa & Edwards, 2006).

Usually, when a resource (such as food, territory, or mating partners) can be controlled and monopolised, group living animals are more likely to establish a dominance hierarchy, and group members must compete to reach a dominant position in the hierarchy (Schjelderup-Ebbe, 1922; Drews, 1993). The establishment and maintenance of a dominance hierarchy encompasses a series of aggressive and submissive interactions between group members: individuals that compete and prevail in most of the conflicts are considered as dominant, and those who lose those conflicts are subordinates (Wingfield & Sapolsky, 2003; Sapolsky, 2005; Zhou et al., 2018; Tibbetts et al., 2022). Dominance hierarchies may also be defined as steep or shallow if we consider a continuous gradient (de Vries et al., 2006; Sánchez-Tójar et al., 2018). In steep hierarchies, the dominant individuals win dyadic contests (that is, no other individual is involved in the conflict) most of the time with subordinate rivals (de Vries et al., 2006). In shallow hierarchies, the outcomes of dyadic conflicts are dependent on the context: the individual “A” may win some conflicts with the individual “B” and lose some other depending on when, where, and for what resource the contest is about (Kadry & Barreto, 2010). Dominance hierarchy establishment is therefore an important factor for group stability, and it also restricts the escalation of aggression: subordinate members are less likely to fight dominant individuals, and at the same time to incur in risks of aggression and injuries (Tibbetts et al., 2022). Dominance hierarchies may either be stable over time or have frequent changes over a short period of time, especially when events such as immigration, emigration, or deaths lead to shifts in the group composition (Neumann et al., 2011). Being a dominant individual in a group has a direct impact on the individual's access to resources, and dominant animals typically gain more benefits (for example, access to food or a mating partner) than subordinates, which also results in a fitness advantage (Cant & Field, 2005).

1.3 Nonapeptides and their role in social interactions

The correlation between hormones and social behaviour has historically attracted the interest of many researchers (Herbert, 1977; Soares et al., 2010; O'Connell & Hofmann, 2011). Broadly speaking, hormones function as regulators of the neural mechanisms that underlie social behaviours (Oliveira, 2009). On the other hand, sociality can affect neuroendocrine mechanisms, leading to changes in hormone levels that, in turn, modulate neural mechanisms and subsequent social behaviours. In essence, hormones can influence behaviour, but they also respond to it (Oliveira, 2004).

Recently, neuroscientists have started investigating the role of nonapeptides in relation to the expression of social behaviours (Caldwell et al., 2008; Donaldson & Young, 2008; Lee et al., 2009; Insel, 2010; Albers, 2012): Nonapeptides are molecules that function both as neurotransmitters and neurohormones, and most of the neuroscientific research on social behaviour has been focused on the oxytocin and vasopressin families, which include oxytocin (OT) and arginine-vasopressin (AVP) in mammals, and the OT and AVP homologues – isotocin (IT) and vasotocin (AVT) – in fishes (Caldwell et al., 2008; Donaldson & Young, 2008; Lee et al., 2009; Insel, 2010; Albers, 2012). The nonapeptides' regulating role of social behaviours appears to be deeply conserved across vertebrates, and homologies are found between mammals (Donaldson & Young, 2008) and fish (Goodson & Thompson, 2010).

AVT is a nonapeptide acting both as neurotransmitter and as neuromodulator in the central nervous system of teleosts (Kulczykowska, 2008; Goodson & Thompson, 2010;

Godwin & Thompson, 2012), birds (Goossens et al., 1977) and amphibians (Moore & Lowry, 1998; Moore et al., 2005). AVT is involved in the control of the hypothalamic-pituitary-interrenal (HPI) axis, and it has been suggested that it may modulate agonistic behaviour in teleost fish (reviewed by (Backström & Winberg, 2017)). Larson et al. (Larson et al., 2006) observed through immunohistochemistry that dominant zebrafish have higher numbers of AVT cells in their brain magnocellular pre-optic area (POA) than of the subordinates, and that magnocellular AVT cells were also larger in dominant than in subordinate zebrafish. However, in the parvocellular POA, the reverse relationship was observed, with subordinate zebrafish having larger and higher numbers of AVT cells than dominant fish (Larson et al., 2006). By contrast, in Mozambique tilapia (*Oreochromis mossambicus*), subordinate (non-territorial) males showed larger AVT cells in the POA than territorial males, and even though the number of POA AVT cells did not differ between non-territorial and territorial males, non-territorials displayed a higher number of gigantocellular AVT cells (Almeida & Oliveira, 2015). The significance of this observation is unclear, even though Almeida and Oliveira suggested that it could be related to an association between the HPI axis and the gigantocellular AVT nucleus. Interestingly, in *Astatotilapia burtoni*, an African cichlid species, territorial males showed higher expression of AVT mRNA in the gigantocellular nucleus than non-territorial males, and AVT mRNA levels correlated with aggressive behaviour (Greenwood et al., 2008). Thus, even though AVT appears related to agonistic behaviour, the role of this peptide in controlling behaviour is likely to vary between species and contexts (Teles et al., 2016). For example, dominant daffodil cichlids (*Neolamprologus pulcher*) have higher brain AVT gene expression than do subordinates (Aubin-Horth et al., 2007), but subordinates have higher levels of free AVT peptide, the biologically active form (Reddon et al., 2015).

1.4 The daffodil cichlid, a model for conflict management

Although much effort has been put in investigating conflict management in primates and other mammals (Aureli & de Waal, 2000), such observations are mostly conducted on unmanipulated animals in the wild, as performing carefully controlled experiments using these species in laboratory settings is often infeasible. *Neolamprologus pulcher*, commonly known as the daffodil cichlid fish, has recently gained momentum as model system in social behaviour research (Wong & Balshine, 2011a). The daffodil cichlid is a freshwater fish species endemic to Lake Tanganyika, Africa, where it inhabits the shallow waters of the lake's Southern coasts (Taborsky & Limberger, 1981; Balshine et al., 1998). These fish form complex permanent social groups to reduce predation risk (Balshine et al., 2001; Heg et al., 2004; Wong & Balshine, 2011a) and they perform a form of sociality which revolves around cooperative breeding (Wong & Balshine, 2011a), a form of social cooperation diffused among different taxa (Jennions & Macdonald, 1994; Arnold & Owens, 1999; Wong & Balshine, 2011a; Cant, 2012; Shen et al., 2017), and characterised by alloparental care, in which offspring receive care and protection from their parents and also from other members of the social group (Lukas & Clutton-Brock, 2012). In daffodil cichlids, a social group is generally composed of a dominant pair, usually the largest male and female fish, and up to 20 smaller subordinate fish (known as helpers) that may either be offspring from previous broods or individuals who join the social group at different stages of their life; only the dominant pair breeds, while both breeders and helpers work to guard and maintain the territory and take care of the offspring of the dominant pair (Balshine et al., 1998; Balshine et al., 2001; Heg et al., 2005; Desjardins et al., 2008).

Daffodil cichlids may be kept in laboratory setting without using excessive space, and the social composition of each group can be easily manipulated. Noteworthy, when housed in aquaria, daffodil cichlids perform their full suite of social behaviours, making them an ideal study system for controlled experiments on sociality (Taves et al., 2009; Le Vin et al., 2010; Bruintjes & Radford, 2013; Dey et al., 2015; Reddon et al., 2015), and in particular they may serve as a model system for studies on conflict management (Balshine et al., 2017). The potential sources of conflict within daffodil cichlid social groups are well-documented: for example, the availability of suitable shelters has been acknowledged as a cause of conflict among dominant individuals (Reddon et al., 2011; Hick et al., 2014). The proximity of subordinate individuals to breeding positions in the group hierarchy is also known to generate competition, and changes in the hierarchy of a social group may induce aggressiveness in individuals ascending in ranks as a way to re-establish dominance relationships (Wong & Balshine, 2011b). Non-dominant individuals in a group may display submission to reduce aggression from dominant group members (Balshine et al., 1998; Bergmüller et al., 2005; Bergmüller & Taborsky, 2005; Balshine et al., 2017). As opposed to fleeing from an aggressor, the submissive individual yields from the conflict avoiding further costly aggressive interactions while remaining within the same spatial location (Ligon, 2014). Subordinate individuals may also avoid aggression by performing cooperative behaviours towards the offspring of dominant individuals as a “pay to stay” strategy, in which help may serve as a form of payment for the privilege of being tolerated within the dominants' territory (Gaston, 1978). Importantly, differential social experiences have been found to affect the behaviours of daffodil cichlids during their development. Fish that grow within a social group with dominant individuals in it display more social competence than individuals growing up without breeders (Arnold & Taborsky, 2010; Taborsky et al., 2012; Fischer et al., 2017). Helpers may also benefit from the protection of the group through the dilution of predation risk, and mutualistic defence against predators and rivals (Balshine et al., 2001; Groenewoud et al., 2016). On the other hand, the presence of helpers may impose costs on breeders in the form of reproductive parasitism, thereby causing increased aggression toward subordinate individuals (Dierkes et al., 1999). As such, understanding the functional processes underlying these group dynamics remains a key challenge in social behaviour research.

1.5 Conclusions

Over the last 30 years, the definition of sociality has been broadened from the simplistic meaning of “group-living” (Majolo & Huang, 2022), and it now encompasses a diverse range of socially relevant interactions and cognitive processes, such as the tendency to approach conspecifics, individual recognition, and discrimination, parental and alloparental care, and conflict management tactics (Goodson, 2013). The emergence of such social behaviours is often the result of the gradual accumulation and refinement of slight behavioural modifications driven by subtle changes in the underlying physiological mechanisms of an animal (Goodson et al., 2005; Donaldson & Young, 2008; Soares et al., 2010; O'Connell & Hofmann, 2011; Zayed & Robinson, 2012). As the simple behavioural building blocks have likely evolved independently in different animal species over hundreds or thousands of generations (Goodson, 2013), it is vital to recognise how they have functionally transformed during the divergence of social systems to comprehend the evolution of complex social behaviour and group living patterns. Likewise, understanding how social animals manage conflicts is a fundamental step towards a comprehensive knowledge of group living.

The main goal of my PhD research project was to identify the behavioural and physiological mechanisms of conflict management in daffodil cichlids. Toward this aim, I followed an innovative and integrated multidisciplinary approach, which encompasses neuroscientific measures, through the use of fluorescent immunohistochemistry, and behavioural observations. Integrative approaches combining classic behavioural techniques, involving group/environmental manipulations, with neurophysiological tools (Taborsky, 2014; Fawcett et al., 2015) have been increasingly adopted to provide a comprehensive portrait of animal sociality (Soares et al., 2010; Bshary & Oliveira, 2015; Taborsky & Taborsky, 2015; Kasper et al., 2017). For example, the way an individual develops has a huge impact on its behaviours and its eventual role in a social group (English et al., 2015), but most of the social vertebrates maintain a behavioural plasticity throughout the course of their life (Kasper et al., 2017), and they may adapt their social roles and behaviours depending on the environmental and social conditions (Bruintjes & Taborsky, 2011), both behaviourally and physiologically.

In particular, in my PhD research project I have focused on the phenotypic expression of aggressive and submissive behaviours during the resolution of conflicts, the function of the environment, and the role of AVT on the regulation of these social behaviours in daffodil cichlids. The daffodil cichlid is an ideal animal model for integrative social behaviour research, as it offers a unique opportunity to conduct controlled behavioural studies in a highly social vertebrate. Therefore, the experimental part of this thesis is composed of four chapters. In the first experimental chapter of this thesis, I focused on examining the function of a peculiar behaviour of subordinate daffodil cichlids, the head up display, to confirm that this signal does indeed serves as a submission signal and therefore a way to pre-emptively resolve conflicts within daffodil cichlids' social groups. In the second experimental chapter, I aimed at understanding the role of the ecological context in the expression of submissive behaviours by manipulating the environment in which daffodil cichlids live in. Next, in the third experimental chapter, I examined the differences in the expression of agonistic behaviours in dominant and subordinate daffodil cichlids, looking at the possible correlations between behaviours and AVT immunoreactive neurons in the preoptic area of the brain. Finally, in the last experimental chapter, my goal was to examine the role of AVT during the establishment of dominance hierarchies in daffodil cichlids after a territorial contest.

Chapter 2: Submissive signalling in subordinate daffodil cichlids.

Tommaso Ruberto, Jamie L. Talbot, Adam R. Reddon

2.1 ABSTRACT

Dominance hierarchies can reduce conflict within social groups and agonistic signals can help to establish and maintain these hierarchies. Behaviours directed towards a dominant individual by a subordinate individual are often assumed to function as signals of submission, however, these behaviours may serve other purposes, for example, defensive or affiliative functions. For a behaviour to act as a submission signal, the receiver must respond by reducing their likelihood of aggression towards the signaller. In the current study, we examine the receiver response to a putative signal of submission, the head up display, within established social groups of the cooperatively breeding fish, the daffodil cichlid (*Neolamprologus pulcher*). We found that when subordinate signallers produce the head up display in response to aggression from the dominant breeder male, the breeder exhibited a longer latency to behave aggressively towards that individual again. We also report that head up displays are rarely produced without being elicited by aggression, and the number of head up displays correlates strongly with the amount of aggression received. Collectively, our results demonstrate that the head up display is used as a signal of submission in the daffodil cichlid. Our findings provide insight into intragroup communication in an emerging model system for the study of social behaviour.

Keywords: aggression, communication, cooperative breeding, fish, Neolamprologus pulcher, submission

2.2 INTRODUCTION

Group living confers many benefits to those within the group, including but not limited to increased safety from predators (Alexander, 1974; Krause & Ruxton, 2002). Yet, within group conflict may impose sufficient costs on some group members (Lorenz, 1966; King, 1973; Krause & Ruxton, 2002) to destabilise social groups or prevent the formation of groups to begin with (de Waal, 1986; Aureli et al., 2002; Silk, 2007; Kutsukake & Clutton-Brock, 2008). Adaptations that mitigate conflict within social groups are a universal feature of animals living in complex social groups (Aureli & de Waal, 2000), in which individuals engage in frequent interactions across various contexts, often maintaining repeated interactions over time with the same individuals pertaining to the same networks (Freeberg et al., 2012). In this context, dominance hierarchies can help to avoid conflict within established social groups by setting priority access to resources without the need for frequent costly aggressive interactions (Rowell, 1974; Bernstein, 1981; Drews, 1993; Wilson, 2000). In order to establish and maintain dominance hierarchies, animals make use of both stable indicators of social status, for example differences in colouration or markings (Cervo et al., 2008; Chen & Fernald, 2011; Dey et al., 2014), and more flexible behavioural indicators of status (Ward & Webster, 2016), with or without individual recognition mechanisms (Dugatkin & Earley, 2004). Signals of aggressive intent or lack thereof can help to stabilise dominance hierarchies, and therefore promote group living (Bernstein, 1981; Frommen, 2020). Submission signals in particular allow subordinate individuals to clearly communicate their lack of motivation to perpetuate or escalate an aggressive interaction against a socially dominant and/or physically superior receiver (Bernstein, 1981; Deag & Scott, 1999; Flack & de Waal, 2007; Petit, 2010). As a result, submission signals are an essential aspect of communication within complex social groups (Schenkel, 1967; Bernstein, 1981; Freeberg et al., 2012; Frommen, 2020), and are widespread throughout the animal kingdom (Judge & de Waal, 1993; Sánchez-Hernández et al., 2012; Balshine et al., 2017; Reddon et al., 2019).

To act as a signal of submission, a behaviour must reduce the likelihood of further aggression from the receiver (Bradbury & Vehrencamp 1998). For example, in contests between veiled chameleons (*Chamaeleo calyptratus*), the losing individual signals their lack of intention to persist by darkening their body colouration. The receiver reacts to this darkening of the body with a precipitous decrease in further aggression (Ligon, 2014). Similarly, several fish species change their body colouration in response to aggression: for example, the common blenny (*Lipophrys pholis*) blanch to signal submission, thereby reducing aggression received (Gibson, 1967). Oscars (*Astronotus ocellatus*) defeated in a contest change their body patterns into a uniform dark colouration (Beeching, 1995), while salmon (*Salmo salar*) darken their body and eye colour to indicate submission (O'Connor et al., 1999), and in both cases this darkening reduces aggression from the receiver. Signallers may use vocalisations or assume a non-threatening body posture, for example by lowering their ears and tail (Fox, 1969; Leyhausen & Tonkin, 1979) to communicate their submission towards an aggressive individual. In fallow deer (*Dama dama*), the lateral display of the antlers, turning the head away from an opponent by the loser of a contest serves to de-escalate the conflict (Jennings et al., 2002). Similarly, in little blue penguins (*Eudyptula minor*), turning the head to look away from an attacker is used as a submission signal (Waas, 1990).

However, not all apparent submissive behaviour actually decreases the aggressiveness of the receiver, at least not in all contexts in which the behaviour is produced. In meerkats (*Suricata suricatta*), females that show the most submissive behaviour also receive the most aggression from the dominant pair and are most likely to be evicted from the social group (Kutsukake & Clutton-Brock, 2006). On the other hand, seemingly submissive behaviours may be used by animals as a defensive tactic during contests to protect a vulnerable part of their body, or even as a way to prepare a counterattack (Pellis & Pellis, 2015). For example, rolling over on to the back and assuming a supine posture, which is a frequent manoeuvre during play fights (Fox, 1969; Bauer & Smuts, 2007), also has an apparent submissive function in dogs and wolves (Lorenz, 1943;

Schenkel, 1967). However, in both dogs (Norman et al., 2015) and wolves (Cordoni, 2009), this behaviour has been found to be more consistently used as a combat tactic than as a submission signal. Similarly, jacky dragons (*Amphibolurus muricatus)* were found to strategically use the same signals to escalate or de-escalate a conflict, depending on the context of the signals produced by their opponent (Van Dyk & Evans, 2008).

The cooperatively breeding cichlid fish *Neolamprologus pulcher*, commonly known as the daffodil cichlid, is a freshwater species endemic to Lake Tanganyika, Africa (Taborsky & Limberger, 1981; Balshine et al., 1998). These small fish form permanent social groups organised into a size-based dominance hierarchy (Wong & Balshine, 2011a). A group of daffodil cichlids is generally composed of a dominant pair, usually the largest male and female fish, and 1-20 smaller subordinate fish of varying size (Taborsky, 1984; Taborsky, 1985; Balshine et al., 2001; Heg et al., 2005; Desjardins et al., 2008). In daffodil cichlid groups, only the dominant pair typically reproduce, while both breeders and subordinates work together to guard and maintain the territory and take care of the offspring of the dominant pair (Wong & Balshine, 2011a). Conflicts and agonistic behaviours among group members are well-documented in this species (Reddon et al., 2011; Reddon et al., 2013; Balshine et al., 2017). Potential sources of conflict within *N. pulcher* social groups include the availability of suitable shelters (Reddon et al., 2011; Hick et al., 2014) and the distribution of workload among group members (Fischer et al., 2014). The proximity of subordinate individuals to breeding positions is also known to generate competition (Dey et al. 2013), and changes in the hierarchy of a social group may induce aggressiveness in individuals ascending in rank as a way to re-establish dominance relationships (Wong & Balshine, 2011b).

Previous empirical and theoretical work has established that movement restrictions correlate with the expression of submission signals (Schenkel, 1967; Aureli & de Waal, 2000; Matsumura & Hayden, 2006; Huntingford & Turner, 2013). Submission is more common when the subordinate animal is unable to easily move away from or physically escape aggression from the dominant individual, for example in chameleons because of their low movement speed (Ligon, 2014). Daffodil cichlids are highly capable swimmers; however, their movements are restricted by their social system, and they are reliant upon their continued membership within the social group for survival due to the high predation pressure in their natural environment (Groenewoud et al., 2016). So, while daffodil cichlids could escape aggression from a dominant fish in the wild, to do so would necessitate leaving the small, sheltered, territory controlled by the group and thus risk being depredated (Balshine et al., 2001; Wong & Balshine, 2011a; Groenewoud et al., 2016). Hence, there is good reason to assume that submission signals ought to be an important aspect of the social repertoire of this species (Balshine et al., 2017; Reddon et al., 2019). Submissive behaviours may prevent aggression from dominants (Bergmüller & Taborsky, 2005; Fischer et al., 2014; Fischer et al., 2017), and increase the likelihood that a subordinate may be accepted as part of the group (Taborsky et al., 2012). Thus, they are likely an important factor in cementing the social groups (Taborsky & Grantner, 1998; Balshine et al., 2017; Fischer et al., 2017).

One of the apparent submission signals displayed by the daffodil cichlid is the head up display (HUD), in which the signalling fish tilts its body upwards in the water column and presents its underbelly to the receiver (Hick et al., 2014). Regarding the HUD itself, the extent to which the fish raises its head in the water column can range from a subtle upward pivot to adopting a nearly perpendicular position. Additional behaviours, like tail or body quivering, may also be observed alongside the HUD (Hick et al., 2014; Reddon et al., 2015). The extent of lateral movement ranges from absence to a subtle quivering of the tail, up to a complete body shake. This phenomenon may be also associated with the body tilt angle, implying that these components may potentially convey information about signal amplitude. Subordinates often respond to aggression with HUDs, suggesting this behaviour acts as a submission signal which may help to mitigate conflict with higher ranking individuals (Taborsky, 1985; Reddon et al., 2012; Hick et al., 2014), and maintain the social hierarchy (Bergmüller & Taborsky, 2005; Balshine et al., 2017; Reddon et al., 2019). The expression of HUDs is strongly predicted by aggression from a dominant fish (Reddon et al., 2019), as expected for a submissive response to an aggressive signal. However, it remains unknown if receiving the HUD actually reduces the likelihood of future aggression.

In the current study, we aimed at confirming that the HUD in the daffodil cichlid does indeed serve as a submission signal within social groups. We predicted that the receiver would reduce the frequency of further aggression by increasing the average latency to the next aggression directed at the signalling individual. We also examined the effect of relative body size on the propensity to produce HUDs in interactions with dominant group members. Based on previous work on submission in this species (Reddon et al., 2019), we predicted that relatively smaller and therefore less threatening individuals would be more likely to show HUDs.

2.3 MATERIALS AND METHODS

2.3.1 Study animals

The daffodil cichlids (*Neolamprologus pulcher*) used in this experiment were laboratory reared descendants of animals captured from the Kasakalawe Point along the southern shore of Lake Tanganyika (Zambia, Africa). Prior to the onset of the study, the subjects were housed in mixed sex stock aquaria (105 x 43cm and 40cm high, 180-litre) at a density of approximately 50 fish per aquarium. These stock aquaria contained 2 internal powered filters, a heater, a thermometer, an air stone, and 3cm of fine coral sand. The stock aquaria were held at 27±1°C on a 12:12h light:dark cycle, with 30 minutes of gradual brightening/dimming to simulate sunrise and sunset. The aquaria were regularly checked for water quality parameters. Fish were fed daily on cichlid flake food (Tetra Cichlid XL Flakes, Tetra Werke, Germany).

2.3.2 Focal groups

We created 10 focal social groups of 4 fish each by transferring fish from the stock aquaria into 90-litre (53 x 43cm and 38cm high) group housing aquaria. Each group consisted of a breeder male (mean ± SE standard length, measured from the tip of the snout to the end of the caudal peduncle = 5.33 ± 0.19cm), a breeder female (mean ± SE standard length = 4.80 ± 0.16), and two smaller subordinates of indeterminate sex. Of the two subordinates, the larger within each group was referred to as “subordinate 1” (mean ± SE standard length = 3.31 ± 0.19cm) and the smaller of the two as “subordinate 2” (mean ± SE standard length = 2.72 ± 0.11cm). Each of the group housing aquaria was furnished with two foam filters, a heater, a thermometer, 3 cm of fine coral sand, along with 4 terracotta caves to serve as shelters and breeding substrate. Two additional floating shelters made from translucent green PET bottles were provided near the surface of the water. The husbandry regime for the social groups was identical to that of the stock housing aquaria.

These groups were formed by first introducing the subordinates into the new aquarium, and then 24h later, adding the breeders. New groups were carefully monitored for excessive aggression or the social rejection of any group members, and unstable groups were dissolved and reformed with new fish. All groups used in this study lived together as a group for at least one month prior to observation and had successfully produced offspring at least once. At the time of observation, all groups contained fry (<1cm standard length). Adult and larger juvenile daffodil cichlids do not typically interact with fry in the social group (Dey et al., 2015).

2.3.3 Experimental procedures

Each group was recorded with a video camera (CX240E Full HD Camcorder, Sony Corp., Japan) from a front-on perspective for five 30-minute periods over the course of two weeks, resulting in a total of 150 minutes of observation per group. The video recordings were captured between 10h and 18h and only one recording was taken per day. Due to the Sars-Cov-19 outbreak, one group was dissolved before the onset of the observations and was subsequentially excluded from the analyses.

Behavioural scoring was performed manually during the experiments by a trained observer (T.R.). For the behavioural coding, we focused on the interactions between the dominant breeder male and the other three group members (breeder female, subordinate 1, subordinate 2). The breeder male frequently showed aggression to other group members and never showed HUDs, consistent with previous reports (Taborsky & Grantner, 1998).

For each group, we recorded every instance of aggression directed by the breeder male to any of the other three group members. We recorded five different behaviours as aggression: chases, rams, bites, head down displays, fin spreads, and frontal displays (for a detailed description of these behaviours, see (Reddon et al., 2015)). We also recorded every instance of a HUD produced by any of the other three group members towards the breeder male.

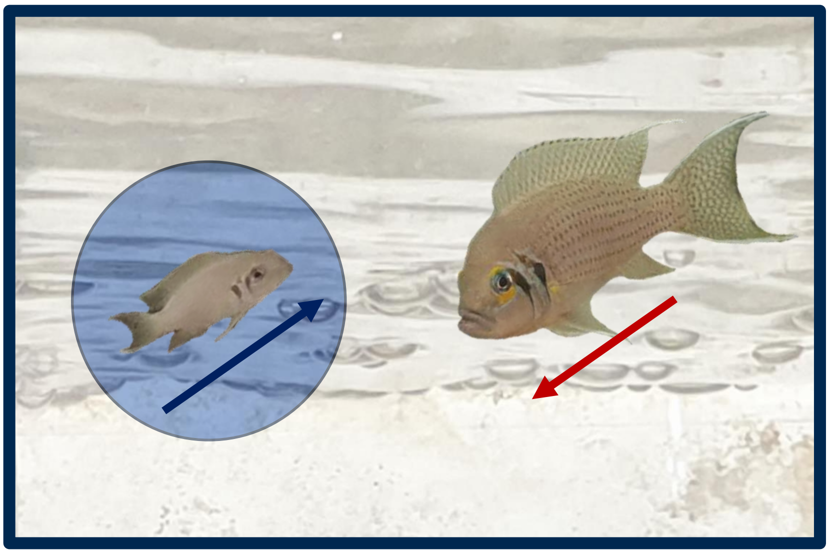

Figure 1. Representation of an event of HUD event from a helper daffodil cichlid (on the left) in response to a head down display from a dominant male (on the right). The blue arrow indicates the helper tendency to tilt its body towards the water surface, presenting its underbelly to the dominant as a sign of submission, while the red arrow indicates the tendency of the dominant to tilt its body downwards to signal a restrained aggression.

Following each act of aggression from the breeder male towards one of the other three group members, we recorded how the receiving fish responded with a HUD. Although the HUDs were accompanied by other submissive behaviours, like tail quivers and/or body shakes, or other temporary morphological changes, as for example body colour changes, for the purpose of this experiment we did not measure these behaviours (see Appendix 2 for a more thorough examination of other behaviours associated with the HUDs). Instead, we chose to focus on the HUDs as a response to aggression received, and we also recorded the latency (in seconds) to the next instance of aggression from the breeder male to that individual.

2.3.4 Statistical analysis

We used a Linear Mixed Model (LMM) to examine the relationship between the number of aggressive behaviours received from the breeder male and the number of HUDs directed at the breeder male. In a separate LMM, we examined the relationship between body size relative to the breeder male (focal standard length/breeder male standard length) and the number of HUDs produced per aggression received (HUD rate). Finally, we used an LMM to examine latency to the next aggression from the breeder male following a HUD compared to aggression that did not elicit a HUD in the focal fish. For this last analysis, we included only fish for which we observed at least two instances of each type of response to breeder male aggression (HUD shown, HUD not shown). This reduced the sample size for this analysis to n = 17 focal fish. We calculated the mean latency to the next aggression from the breeder male after each type of response for each fish and treated response type as a repeated measure within each focal individual. We log10 transformed the mean latency to the next aggression prior to analysis to account for the positive skew in this data but present the raw data graphically. For all three models, social group was included as a random factor. We checked all models for adherence to model assumptions by examining the Q-Q plots of the model residuals. All statistics were performed using SPSS version 26.0 (IBM) for Macintosh (macOS 10.15.4).

2.3.5 Ethical note

Animal housing, handling, and study protocols were approved by the Liverpool John Moores Animal Welfare and Ethics Steering Group (approval number: AR_TR/2018-4) and adhered to the guidelines of the Animal Behaviour Society and the Association for the Study of Animal Behaviour. All fish were closely monitored for social exclusion or signs of injury. All observations were drawn from stable social groups showing typical levels of agonism for daffodil cichlids (Balshine et al., 2017).

2.4 RESULTS

Across the 27 focal fish in 9 groups, we recorded 493 instances of HUDs directed towards the breeder male in 1350 total minutes of observation. The vast majority (94.5%) of HUDs directed towards the breeder male were performed in direct response to aggression.

There was a positive linear relationship between the aggression directed towards each focal fish by the breeder male and the number of HUDs performed to him by those fish ($F_{1,20.45} = 102.11$, $p < 0.001$; Fig. 2). Fish that were relatively smaller than the breeder male showed a greater HUD rate than those that were closer in size ($F_{1,17.76} = 5.96$, $p = 0.025$; Fig. 3).

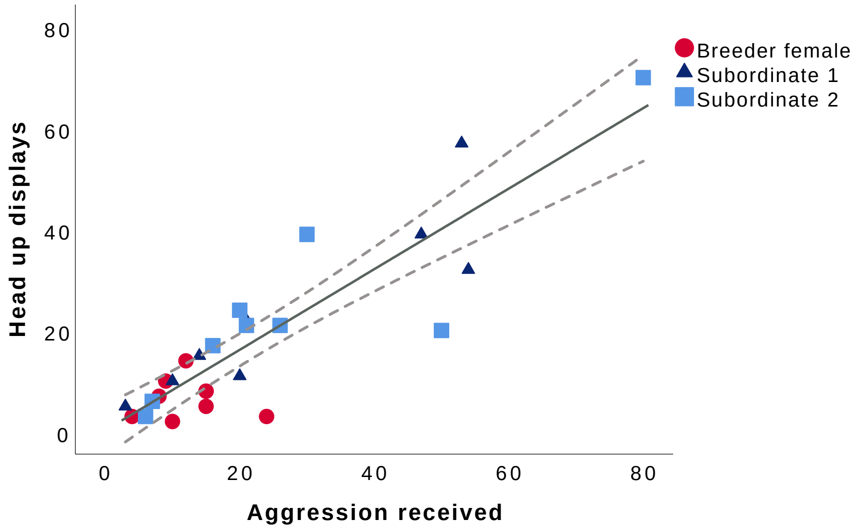

Figure 2. The number of head up displays directed at breeder males as a function of breeder male aggression towards each fish. There is a positive linear relationship (±95% CI; p < 0.001).

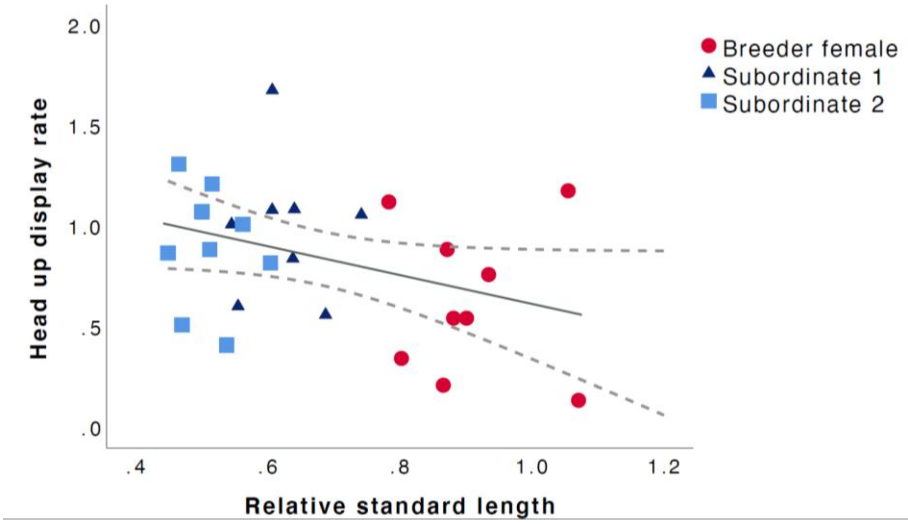

Figure 3. The number of head up displays directed at the breeder male per aggression received (HUD rate) as a function of the relative size of the focal fish to the breeder male (breeder male standard length / focal fish standard length). There is negative linear relationship (±95% CI; p = 0.025).

Focal fish performed at least one HUD after aggression from the breeder male in 401 out of 611 instances (65.6%). The latency to the next aggression from the breeder male was greater when the receiving fish performed a HUD than when they did not ($F_{1,18.71} = 5.38$, $p = 0.032$; Fig. 4).

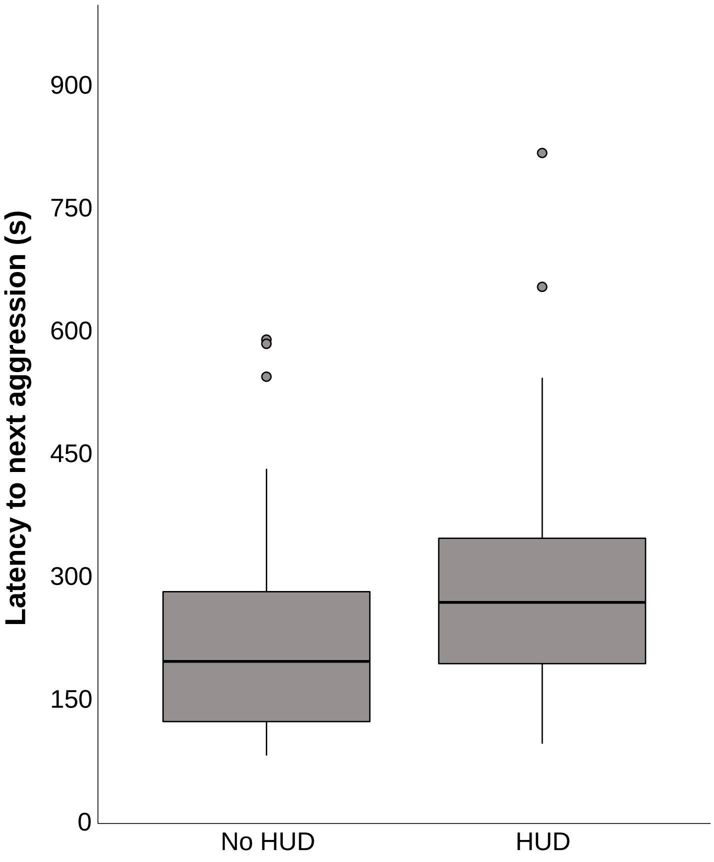

Figure 4. The median latency in seconds for the breeder male to next show aggression to the focal fish following aggression that elicited a head up display compared to aggression that did not. The time to the next aggression was greater when a head up display was produced (p = 0.032).

2.5 DISCUSSION

Using detailed observations of replicate laboratory housed social groups of daffodil cichlids, we found that the head up display (HUD) is given in response to most of the aggression performed by the most dominant group member and is seldom produced in the absence of aggression. We confirmed that the HUD serves as a signal of submission in this species, as has long been assumed (e.g., (Taborsky, 1984; Grantner & Taborsky, 1998; Bergmüller & Taborsky, 2005; Reddon et al., 2019)). Specifically, we found that when receiving aggression from the dominant breeding male, the time until the next instance of aggression depends on whether the receiving fish produces a HUD in response. When the receiver of aggression responds with a HUD, there is a longer average latency to the next instance of aggression from that dominant individual. The HUD may therefore avoid conflict, possibly by communicating a lack of motivation in the signaller to perpetuate or escalate an aggressive interaction. This submissive communication may be beneficial for both the sender and receiver as aggression is costly to all parties in terms of time, energy, and divided attention (Neat et al., 1998; Maan et al., 2001; Copeland et al., 2011).

Occasionally, the focal fish in our study appeared to produce HUDs towards the dominant male without an obvious inciting aggressive act. It is possible that daffodil cichlids do occasionally produce HUDs outside of a submissive context, for example, they may also be used as an affiliative gesture by lower ranked individuals toward those of higher rank. Affiliative and submissive behaviour share common proximate mechanisms in this species (O'Connor et al. 2016). Another possibility is that the apparently spontaneous HUDs we observed were in fact in response to subtle or obscured aggression from the breeder male (for example from behind a shelter out of view of the video recording). Perhaps HUDs are also occasionally shown after a longer delay following aggression, making them appear to be part of a distinct social interaction, or are given pre-emptively to avoid future aggression.

Fish that were much smaller than the breeder male produced more HUDs per aggression received than fish that were closer in size. This fits with previous observations in this species (Reddon et al., 2019), and suggests that HUDs are used more when the signaller poses little threat to the receiver (highly asymmetrical interactions), rather than to clarify or reinforce uncertain dominance relationships (Matsumura & Hayden, 2006).

Previous work in daffodil cichlids has suggested that submission is a key element of social competence (Bergmüller et al., 2005; Taborsky et al., 2012; Fischer et al., 2017), therefore, effective use of HUDs ought to be a characteristic of socially successful individuals (Fischer et al., 2017). Our current findings support this contention. Individuals that show more HUDs receive less aggression from dominant group members, which should allow them more time and energy for other endeavours, such as growth or preparation for future reproduction (Sopinka et al., 2009; Hellmann et al., 2016). It would be informative to examine the future fitness fortunes of individuals that vary in their tendency to deploy HUDs in conflicts with dominant group members.

In some instances, fish do not show HUDs in response to dominant aggression, despite the apparent benefit of doing so. Little is yet known about the context specificity of submissive signal use in this species. Although HUDs appear to be beneficial, submissive behaviour does carry an energetic cost in this species (Grantner & Taborsky, 1998; Taborsky & Grantner, 1998), and in some scenarios it may be advantageous to flee or avoid aggression, rather than showing submissive behaviour. Balshine et al. (2017) found there was a strong negative correlation between the likelihood of showing HUDs and the tendency to flee from an aggressor, suggesting these are alternative strategies for avoiding conflict with stronger opponents. Some individuals may specialise in one tactic over the other, while others tend to use both interchangeably, possibly depending on the context (e.g., (Maestripieri & Wallen, 1997)). Individuals may also pursue heterogeneous strategies which involve the use of multiple distinct visual signals. For example, daffodil cichlids can flexibly display black stripes or chevrons on their opercula as a visual threat signal and a badge of status (Balzarini et al., 2017; Culbert & Balshine, 2019). Although it is still unknown how quickly the intensity of these markings can be altered, it is plausible that daffodil cichlids may supress these markings as a visual signal of submission, similar to the body blanching or darkening of other fish species (Beeching, 1995; O'Connor et al., 1999). It would be interesting to examine the relationship between HUD usage and the expression of these markings to determine the effect these different communication channels have on the receiver.

There is also some variation in the expression of the HUD itself: for example, the degree to which the head is raised in the water column varies from a subtle pivot upwards to the fish assuming a nearly perpendicular position. Other behaviours, such as tail or body quivering, may also accompany the HUD (Reddon et al., 2015), and the degree of lateral movement varies from being absent, through a gentle quivering of the tail, to a full body shake. This appears to correlate with the angle of the tilt in the water (A. R. pers. obs.), suggesting these elements could combine to indicate signal amplitude. In our current study, we did not measure variation in expression of HUDs, and future work examining the meaning of variation in HUDs would be worthwhile. If HUDs do differ in their strength, it would be interesting to investigate under what conditions this variation is expressed. Does the receiver phenotype or the escalation level of the aggression received influence the expression of HUDs? Does social context, such as the presence of potential eavesdroppers affect the expression of HUDs? Alternatively, the expression of the HUD may depend on the signalling environment as more conspicuous or vigorous HUDs may increase signal transmission efficiency in a noisier or more complex signalling situation (e.g., (Eaton & Sloman, 2011; Bruintjes & Radford, 2013)). These questions will need to be addressed in future studies to fully understand the complexity of agonistic communication in this highly social vertebrate.

Our current findings demonstrate that the head up display in the daffodil cichlid acts as a submission signal and reduces the frequency of aggression from the receiver. This behaviour has long been assumed to have this function, but to our knowledge, this is the first demonstration that the HUD has this effect on the receiver. It is essential to understand communication within groups in order to understand the behaviour of social species (Frommen, 2020). Our results help to elucidate the nuances of agonistic signalling in this emerging model for the study of sociality and suggest future avenues for work on the communication system of this species.

Chapter 3: Submissive behaviour is affected by territory quality in a group living cichlid fish.

Tommaso Ruberto, William T. Swaney, Adam R. Reddon

3.1 ABSTRACT

Group living may engender conflict over reproduction or other resources and as such social groups must be able to manage conflict to persist. Submissive behaviours are an adaptation for establishing and maintaining dominance relationships, allowing an individual to relent and avoid further costly aggressive interactions. In the daffodil cichlid fish (*Neolamprologus pulcher*), subordinates may use submissive behaviours to resolve conflicts with dominant individuals and maintain the social status within the group, while remaining in the same spatial location. However, little is known about the effect of the physical environment on submissive behaviours in daffodil cichlids. Here, we investigated how the ecological context affected the expression of submissive behaviours in subordinate daffodil cichlids by manipulating the environment. We systematically altered the number of shelters provided to our experimental groups, and we evaluated the difference in the interactions between dominant and subordinate individuals by scoring the agonistic behaviours of the group members. We found that the aggression levels of the breeders and the submissive and fleeing behaviours of the subordinates were modulated by the environment, with breeders being more aggressive and subordinates displaying fewer submissive behaviours in enriched environments than in any other condition. We also found that larger subordinates were more submissive and smaller helpers were more prone to flee when they responded to the aggression of breeders. Collectively, our results elucidate the role of the physical environment in the regulation of agonistic behaviours and social interactions in this group-living cichlid.

Keywords: daffodil cichlids, aggression, submission, shelters, Neolamprologus pulcher

3.2 INTRODUCTION

For social animals living in complex groups, competition for resources may be frequent and have substantial costs (Aureli & de Waal, 2000; Hardy & Briffa, 2013). Managing within-group competition is therefore crucial for the stability of social groups (de Waal, 1986; Aureli et al., 2002; Silk, 2007; Kutsukake & Clutton-Brock, 2008). Many social animals include within their group offspring and/or related individuals with common inclusive fitness interests (Hamilton, 1964; Eberhard, 1975; Lehmann et al., 2007), and even when there is no relatedness among the members of the group, individual fitness can be significantly enhanced by group productivity (Kokko et al., 2001). Despite such shared interests in complex social groups, competitive interactions are frequent and may range from agonistic displays at a distance to physical contact, and the winner of the competition may have priority or exclusive access to the disputed resource (Kaufmann, 1983). However, agonistic interactions are costly, in terms of resources and time spent performing such interactions, and physical contacts may result in injuries, or even death for both the winner and the loser of the interaction (Forkman & Haskell, 2004; Beaulieu et al., 2014).

To reduce or avoid such costs, many social species exhibit submissive behaviours. Submissive behaviours include both avoidance behaviours such as fleeing from the aggression, and submission signals which are primarily used by animals to communicate submission towards an aggressive conspecific (Reddon et al., 2022). Although avoidance and submission signals are usually mixed into the same category of social behaviours, the evolution, the causes, and the consequences of such behaviours can be distinct. While avoidance behaviours are used to evade aggression and may secondarily serve as a cue of submission, submission signals are not intrinsically linked to escape but rather are primarily a communicative action for example a shift in body colouration or a particular vocalisation (Guilford & Dawkins, 1995). Therefore, the value of a submission signal depends on its impact on the aggressor’s behaviour (Reddon et al., 2022).

To understand how conflict is managed within complex social groups, it is vital to comprehend the factors that influence the use of aggressive and submission signals within the group. The structure of the physical environment is a crucial ecological factor which can influence competition for resources and may determine whether a social group will thrive or fail (Bell et al., 2012). There is growing evidence of widespread differences in social dynamics, occurring within species depending on events that modify the environment in which the animals live in (Brown, 1971; Ekman, 1987; Snell et al., 1988; Anholt, 1990; Ward & Porter, 1993; Petren & Case, 1998; Cross et al., 2004; Wittemyer et al., 2005; Estevez et al., 2007; Henzi et al., 2009; de Silva et al., 2011; Godfrey et al., 2013; Pinter-Wollman et al., 2014; Smith-Aguilar et al., 2016). For instance, an increase in temperature is positively correlated with aggressive behaviours in several fish species (Kvarnemo, 1998; Biro & Stamps, 2010; Zhao & Feng, 2015), and in some cases, temperature rise may affect boldness and inter-individual distances (Angiulli et al., 2020). It is well-known that the availability of resources also has an influence on social behaviour: for example, in the Japanese medaka (*Oryzias latipes*), individuals are more socially tolerant towards other group members and consume less energy in conflict when food is abundant in their territory; on the other hand, when food becomes scarce, individuals increase their aggressiveness (Magnuson, 1962). Therefore, environmental changes that affect the availability of resources, such as seasonality (dry versus rainy seasons) or stochastic events (e.g., storms, drought), may also give rise to social behaviour changes.

The cooperatively breeding daffodil cichlid fish (*Neolamprologus pulcher*) is a freshwater fish species endemic to Lake Tanganyika, Africa, where it inhabits the shallow waters of the lake's Southern coasts (Taborsky & Limberger, 1981; Balshine et al., 1998). These small fish form complex permanent social groups to reduce predation risk (Wong & Balshine, 2011a). A group of daffodil cichlids is generally composed of a dominant pair, usually the largest male and female fish, and up to 20 smaller subordinate fish, known as "helpers", that work to guard and maintain the territory, and take care of the offspring of the dominant pair (Taborsky, 1984; Taborsky, 1985; Balshine et al., 2001; Heg et al., 2005;

Desjardins et al., 2008). Among daffodil cichlids, subordinate individuals (helpers) may avoid aggression from dominant individuals by performing cooperative behaviours (Wong & Balshine, 2011a), directly escaping from aggression, or by displaying submission signals (in the form of head-up submissive postures or tail quivering) towards aggressing groupmates (Taborsky, 1985). Disengaging from a conflict and fleeing to a new location should be the easiest strategy for an animal to escape the aggressor, however, for daffodil cichlids retreat is not always a viable option. The vital protection from predation risk provided by the social group may restrict the ability to flee from aggression by limiting the physical area in which an individual may flee to without venturing too far from the group territory (Wong, 2010; Hick et al., 2014; Balshine et al., 2017). On top of these reasons, other ecological limitations on movement, such as a saturated habitat or the absence of a safe location within reach, may hinder their ability to escape (Wong, 2010; Batista et al., 2012). As a result, submission signals are expected as a particularly important aspect of social interactions in daffodil cichlids, especially when fleeing options are limited.

Moreover, in daffodil cichlids, helpers tend to show more submission signals towards the dominant individuals when the group's territory has fewer available refuges due to a decreased opportunity to escape an aggression (Reddon et al., 2019). However, it remains unclear how changes in the physical environment, for example the addition or loss of territory structure, which in the wild can occur due to physical or social processes, may affect the expression of submissive behaviour within established daffodil cichlid groups. As such, we investigated how the manipulation of the physical environment in which daffodil cichlids live may affect the expression of their submissive behaviours. Specifically, we observed the behaviour of established social groups after manipulating the number and the type of available shelters, that is, breeding caves and floating shelters, using a within groups repeated measures design to examine the effects of environmental enrichment on the social behaviour of daffodil cichlids. We predicted that a greater availability of floating shelter would decrease the use of submission signals to de-escalate conflicts and increase the use of fleeing behaviour which may be more successful with more shelter options available. We also examined how changing shelter availability affected dominant breeder aggression towards helpers in the group and helper investment in cooperative workload in order to develop a more complete picture of how changes in the physical environment affect social groups.

Furthermore, based on the results from Chapter 2, we predicted that fish significantly smaller than the breeder male would produce a higher number of submission signals per aggression received compared to fish that were in closer size, as observed in a previous study within this species (Reddon et al., 2019). On the other hand, fish close in size may have less certainty about their relative fighting ability, thereby increasing the risk of an escalated conflict (Enquist & Leimar, 1990). In this perspective, we may expect that the larger helpers will receive increased levels of aggression from the breeders with respect to the smaller ones, thus they will have greater need for submission and/or avoidance in response to the aggressive behaviours received (Matsumura & Hayden, 2006). Finally, based on previous work on submission in this species (Reddon et al., 2019), individuals who are relatively smaller and perceived as less threatening should receive less aggression, therefore we may expect a difference between the two helpers in the aggressions received from the breeders.

3.3 MATERIALS AND METHODS

3.3.1 Study animals

The daffodil cichlids (*N. pulcher*) used in this experiment were laboratory reared descendants of fish originally captured on the southern shore of Lake Tanganyika (Zambia, Africa). Prior to the onset of the study, subjects were housed in mixed sex stock aquaria (105 x 43cm and 40cm high, 180-litre), at a density of approximately 50 fish per aquarium. The stock aquaria were equipped with 2 internal powered filters, a heater, a thermometer, an air stone, and 3cm of fine coral sand. These aquaria were kept at 27±1°C on a 12:12h light:dark day cycle, with 30 minutes of gradual brightening/dimming to replicate sunrise and sunset. The aquaria were regularly checked for water quality parameters and weekly cleanings were performed. Fish were fed daily with a mix of cichlid dry food consisting of flakes and pellets (Tetra Werke, Germany).

3.3.2 Focal groups

We created 10 focal social groups of 4 fish each. Each group consisted of a breeder male (mean ± SE standard length, measured from the tip of the snout to the end of the caudal peduncle = 5.25 ± 0.17cm), a breeder female (mean ± SE standard length = 4.69 ± 0.19), and two smaller helpers of indeterminate sex. The larger of the two helpers within each group was referred to as “helper 1” (mean ± SE standard length = 3.31 ± 0.16cm) and the smaller of the two as “helper 2” (mean ± SE standard length = 2.68 ± 0.10cm). To form new groups, fish from the stock aquaria were transferred into 90-litre (53 x 43cm and 38cm high) group housing aquaria. Groups were formed by first introducing the two helpers into a new aquarium, and after 24h adding the breeders. Each of the group housing aquaria was furnished with two foam filters, a heater, a thermometer, 3 cm of fine coral sand along with 2 terracotta caves to serve as shelters and breeding substrate. New groups were observed for excessive overt aggression or the social rejection of any group members, and unstable groups were dissolved and reformed anew with other fish from the stock aquaria. All groups used for these experiments were kept together for at least one month prior to observation and had successfully spawned at least once. At the time of observation, all groups contained fry (<1cm standard length). Adult and larger juvenile daffodil cichlids in our social groups did not interact with fry, as reported for this species (Dey et al., 2015). The husbandry regime for the social groups was identical to that of the stock housing aquaria.

3.3.3 Experimental procedures

To evaluate the effect of the physical environment on daffodil cichlids, we systematically manipulated the number of available shelters by placing additional floating shelters (PET bottles affixed near the water surface) and additional substrate level shelters (terracotta caves) in the housing tanks using a 2x2 design (the standard aquarium setup, extra caves, extra floating shelters, and both types of extra shelter). Each social group was randomly assigned to an initial experimental condition: Standard setup (2 caves+0 floating shelters, 2C+0F, Fig. 1A), additional caves (4C+0F, Fig. 1B), additional floating shelters (2C+2F, Fig. 1C), and additional caves with additional floating shelters (4C+2F, Fig. 1D).

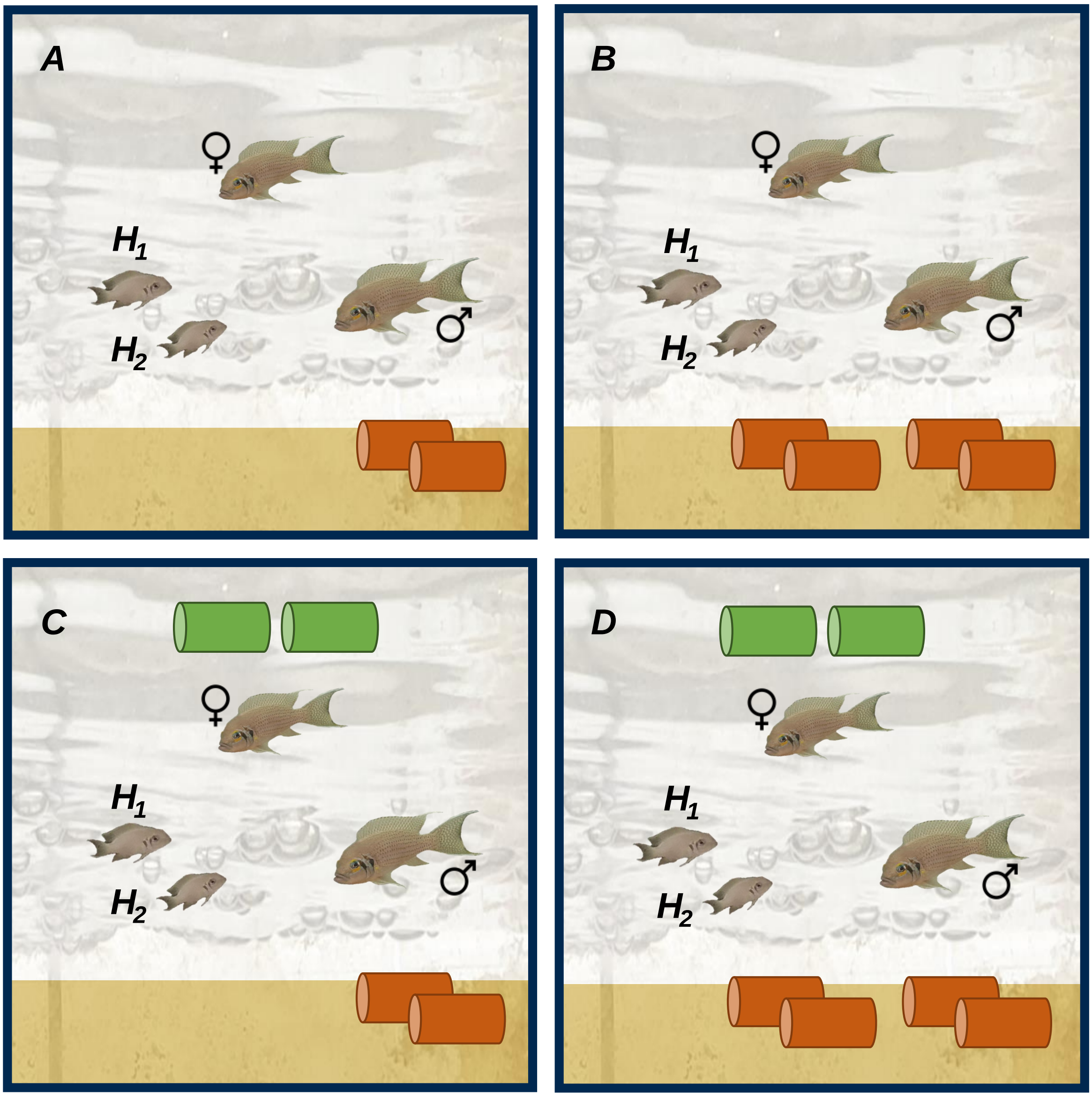

Figure 1. Representation of the daffodil cichlid social groups, with the dominant female (♀) and male (♂), and the two helpers (H_1 and H_2). The figure shows the social groups in the experimental setup for the four conditions examined in our experiments: (A) standard aquarium setup (2 caves+0 floating shelters, 2C+0F); (B) additional caves (4C+0F); (C) additional floating shelters (2C+2F); and (D) additional caves with additional floating shelters (4C+2F).

The additional floating shelters were made from transparent green PET half-bottles and affixed to the aquaria near the surface of the water. In each condition, each group was kept for a week of habituation prior to observation. After the habituation period, we observed each group for ten 30-minute periods over the course of two weeks, resulting in a total of

300 minutes of observation per group per condition. The observations were taken by a stationary observer seated approximately 1.5m from the front of the aquaria. A 10-minute habituation period prior to the onset of coding allowed the fish to acclimate to the presence of the observer. The observations were performed between 10h and 18h and only one observation was taken per day. After the last observation was performed, we changed the treatment condition for the social group by adding or removing caves and/or floating shelters and we gave the group another week of habituation to the new experimental condition prior to observation in the new condition. The order of presentation of the treatments was randomized for each group.

Behavioural scoring was performed manually during the experiments by a trained observer (T.R.). During the observations, we recorded interactions between each of the four group members (breeder male, breeder female, helper 1, helper 2). We focused on aggression received by the two helpers from the breeder male and the breeder female, and the submissive responses of the helpers. We recorded five different behaviours as aggression: chases, rams, bites, head down displays, and frontal displays. The submission signals recorded were head-up displays and tail quiver displays, and any instances of fleeing (Ruberto et al., 2020; Manara et al., 2023). Finally, we recorded any instance of territory maintenance performed by the helpers, both digging inside and outside of the caves, as workload behaviours. For a detailed description of these behaviours, see (Reddon et al., 2015).

Due to the SARS-CoV-2 outbreak and subsequent lockdown in the United Kingdom, our experiments were disrupted. All groups were tested for the experimental condition 2C+2F, however 2 groups were not tested in the conditions 2C+0F and 4C+0F, and one group was not tested in condition 4C+2F, resulting in a sample size of 8 groups for conditions 2C+0F and 4C+0F and 9 for condition 4C+2F.

3.3.4 Statistical analysis

We used Linear Mixed Models (LMMs) focusing on the responses of the helpers to aggression across the four treatments. First, we checked our datasets for any possible violations of statistical assumptions (that is, a normal distribution of residuals and homogeneity of variance across treatments) and proceeded to Box-Cox transform the data when necessary. Next, we evaluated differences between the two helpers in the aggression received from the breeders depending on the experimental condition in an LMM. Then, we coded either the number submission signals (head up displays + tail quivering displays) per aggression received, fleeing per aggression received, or workload performed per aggression received by the helpers as the dependent variable of our LMMs. In all our LMMs, individual identity and group identity of the fish were included as random factors, while rank of the fish (that is, helper 1 or 2) and experimental condition were included as fixed factors. In case of significant effects, a Sidak post hoc test for differences of means was used for pairwise comparisons. All statistics were performed using SPSS version 26.0 (IBM) for Windows.

3.3.5 Ethical note

Animal housing, handling, and study protocols were approved by the Liverpool John Moores Animal Welfare and Ethics Steering Group (approval number: AR_TR/2018-4) and adhered to the guidelines of the Animal Behaviour Society and the Association for the Study of Animal Behaviour. All fish were closely monitored for social exclusion or signs of injury. All observations were drawn from stable social groups showing typical levels of agonism for daffodil cichlids (Balshine et al., 2017).

3.4 RESULTS

The total instances of aggression received from the helpers varied depending on the experimental condition ($F_{3,\ 51.15} = 6.88$, $p = 0.001$; Fig. 2A). In particular, the helpers received more aggression in condition 4C+2F when compared to all the other conditions in post-hoc analyses (see Table 1). The submission signals ($F_{3,\ 53.9} = 10.99$, $p < 0.001$; Fig. 2B) and the fleeing ($F_{3,\ 52.68} = 6.87$, $p = 0.001$; Fig. 2C) observed in the helpers in response to the aggression received also varied depending on the shelter condition. Helpers were less submissive in condition 4C+2F when compared to all the other conditions in post-hoc analyses (see Table 1). With respect to the fleeing in response to the aggression received, the helpers were fleeing less in condition 4C+2F when compared to condition 4C+0F and 2C+2F, but not when compared to condition 2C+0F, in post-hoc analyses; instead, fish fled more in condition 4C+0F when compared to condition 2C+0F in post-hoc analyses. Workload behaviours did not vary across conditions ($F_{3,\ 40.86} = 1.20$, $p = 0.321$; Fig. 2D).

Table 1. Post-hoc results of the analysis performed.

Experimental conditions		Aggression received	Submission signals per aggression received	Fleeing per aggression received	Workload per aggression received
2C+0F	*vs* 4C+0F	0,999	0,661	0,006	0,194
	vs 2C+2F	1,000	0,563	0,129	1,000
	vs 4C+2F	0,003	0,000	1,000	0,794
4C+0F	*vs* 2C+0F	0,999	0,661	0,006	0,194
	vs 2C+2F	1,000	1,000	0,763	0,339
	vs 4C+2F	0,009	0,001	0,002	0,926
2C+2F	*vs* 2C+0F	1,000	0,563	0,129	1,000
	vs 4C+0F	1,000	1,000	0,763	0,339
	vs 4C+2F	0,001	0,001	0,046	0,924
4C+2F	*vs* 2C+0F	0,003	0,000	1,000	0,794
	vs 4C+0F	0,009	0,001	0,002	0,926
	vs 2C+2F	0,001	0,001	0,046	0,924

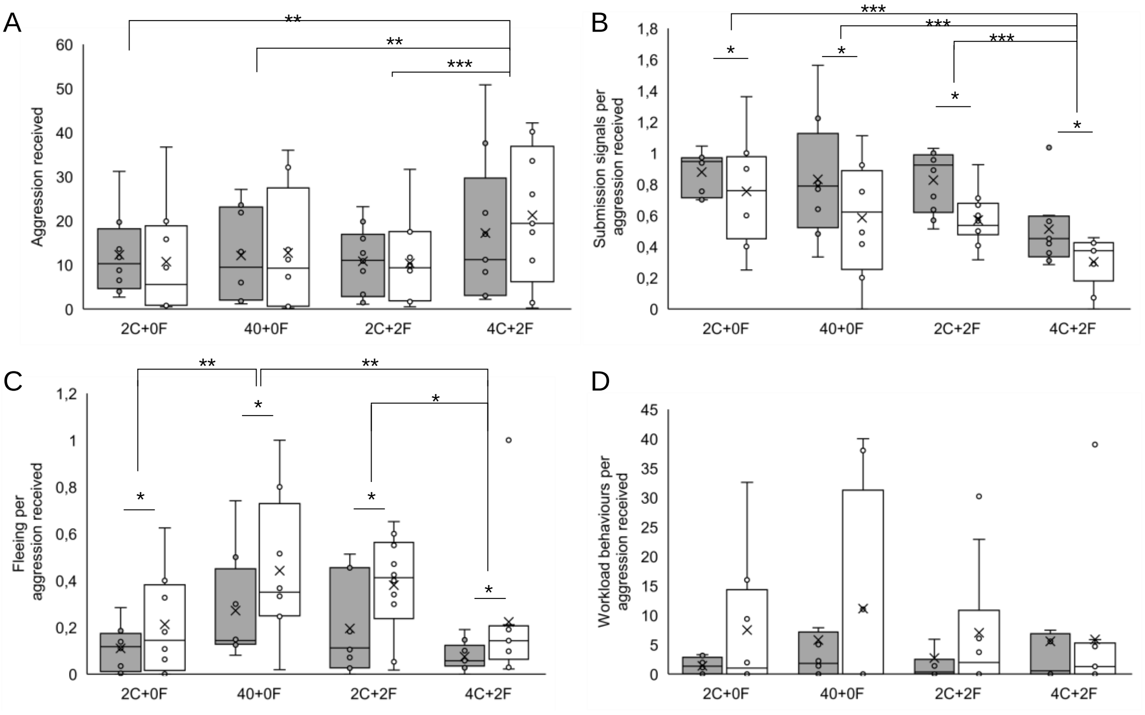

*Figure 2. The aggression received (A), and the submission signals (B), the fleeing (C) and the workload (D) as function of the aggression received by the helper 1 (grey bars) and by the helper 2 (white bars) in the four experimental conditions: standard setup (2C+0F), additional caves (4C+0F), additional floating shelters (2C+2F), and additional caves with additional floating shelters (4C+2F). The * indicates a p < 0.05, ** indicates a p < 0.005, *** indicates a p < 0.001.*

Across all shelter treatments, the number of aggressions received did not differ between the two helpers ($F_{1,\ 20.39}$ = 0.01, p = 0.942). We did find a difference between the two helper ranks in the number of submission signals ($F_{1,\ 10.74}$ = 7.249, p = 0.021) and the fleeing ($F_{1,\ 20.98}$ = 4.66, p = 0.043) performed in response to the aggression received, with the helper 1 performing more submission signals and fewer fleeing bouts with respect to the helper 2. However, no differences were found between the two helpers in the workload performed in response to the aggression received ($F_{1,\ 11.77}$ = 0.01, p = 0.939).

3.5 DISCUSSION

In this study, we investigated how the manipulation of the physical environment in which daffodil cichlids live may affect the expression of their submissive behaviours. We predicted that increasing the territory value would make the breeders more aggressive towards the helpers, which in turn would be more prone to show submissive behaviours and/or workload behaviours to appease the breeders. Alternatively, as the breeders would need more help in maintaining a more complex territory, they could become more tolerant towards helpers who in turn show fewer submissive behaviours and/or fleeing. Increasing territory value may also motivate helpers to retain their position within the group, as the territory would be more valuable upon ascension to the dominant breeding position. We also expected that submissive behaviours would be less common when more places to flee to were available. The results of our experiments showed that the aggression received by the helpers from the breeders varied depending on the experimental condition, with fish receiving more aggression in the most enriched condition. With respect to the workload of the subordinate individuals, we did not find any differences depending on the aggression received from the breeders nor was workload affected by the experimental conditions. Instead, both the submissive behaviours and the fleeing of the subordinate fish differed depending on the aggression received which was affected by the shelter treatment. Finally, although we found no differences between the two subordinate fish in the levels of aggression received, they responded differently to aggression, with the larger subordinates submitting more but fleeing less than the smaller subordinates.

It has been suggested that environmental enrichment can be beneficial in decreasing aggressive behaviour and physiological stress in fish held in captivity while at the same time enhancing their well-being (Gerber et al., 2015; Näslund & Johnsson, 2016). The most widely used type of environmental enrichment is physical enrichment, which involves introducing various objects like physical structures, plants, and substrates into the fish's housing environment to increase its complexity (Johnsson et al., 2014). Several studies have been conducted to investigate the effect of enrichment on fish aggression, but the findings have been mixed, with some studies reporting positive results (Barley & Coleman, 2010; Kadry & Barreto, 2010; Torrezani et al., 2013; Bilhete & Grant, 2016; Xi et al., 2017; Arechavala-Lopez et al., 2019), some reporting negative results (Barreto et al., 2011; Bhat et al., 2015; Kochhann & Val, 2017), and others reporting no effects (Hoelzer, 1987; Kemp et al., 2005; Lachance et al., 2010). These inconsistencies may be linked to differences in species-specific effects, developmental stage, enrichment mode (type, level, and colour), and methodological variations. In our experiments, we found that breeders increased the levels of aggression towards the subordinate fish when they were tested in the most enriched condition. As territorial availability is often a cause of conflict among daffodil cichlids (Reddon et al., 2011; Hick et al., 2014), it is plausible that the increased number of refuges and the more complex environment in which the groups were living was perceived by the breeders as a territory of high value. This, in turn, would increase their aggressiveness for reinforcing their ranks, while at the same time policing the subordinates and coercing them into being more cooperative (Zöttl et al., 2023).

In line with the results from a previous study (Reddon et al., 2019), we found that subordinate fish in the most enriched environment tended to respond to aggression with fewer submissive behaviours than in any other condition. Previous findings suggest that submission should be more common when the opportunity to flee from an aggressor is limited, either by physical or ecological restrictions such as the lack of shelters (Matsumura & Hayden, 2006; Ligon, 2014). As submission and avoidance are alternative strategies for solving conflicts in daffodil cichlids (Balshine et al., 2017), it is plausible that, with additional shelters placed in the environment and more places to hide from the aggressive breeders, the subordinate fish would have less necessity to perform submissive behaviours.

We found that helpers in the most enriched condition were less likely to flee than helpers in the conditions with either only increased caves or only added floating shelters, but they were not different from the most barren condition. Shelters and caves were added to the environment to serve as physical structures for both refuge and breeding substrate. These structures, and in particular the floating shelters, may have partitioned the experimental tank into multiple separated spaces, limiting visual contact among fish, and reducing the frequency of social encounters, thereby potentially lowering instances of aggression. These “safe havens” might have had a detrimental effect on our groups, as the helpers hiding in them may have been perceived by the breeders as idling and not actually being cooperative/helpful for the group. Indeed, in daffodil cichlids and other cooperatively breeding animals, being idle is punished with increased aggression from dominant individuals (Mulder & Langmore, 1993; Balshine et al., 1998; Fischer et al., 2014). Although it could be argued that neglecting the helping duties could be evaluated as the inverse of the workload measure, in our experiments we could not reliably quantify these idling behaviours, so we cannot exclude that fish were avoiding the aggression of the breeders by taking refuge in the floating shelters or behind any other tank furniture.

Territorial fish have an innate inclination to defend a particular area and compete for its resources (Zhang et al., 2019) and it is known that environmental enrichment may lead to increased aggression among fish (Lee & Berejikian, 2009; Barreto et al., 2011). The additional physical structures introduced into the environment could have been perceived by both the breeders and the helpers as a desirable resource, as the territory would have more places to hide from predators and be more valuable in the context of ascension to the dominant breeding position. As such, to preserve their status within the group, subordinates should have increased the use of either submissive behaviours to de-escalate aggressions or fleeing from the aggressors to disengage from the agonistic interactions (Reddon et al., 2022). Moreover, the workload was not impacted by the manipulation of the physical environment, further suggesting that helpers in our experiments may have preferred a strategy of avoidance rather than using workload and/or submissive behaviours to de-escalate aggressions.

Contrary to the results found in Chapter 2, we found that helpers with greater differences in body size with respect to the breeders exhibited fewer submissive behaviours than larger and high-ranked helpers. Conversely, low ranked helpers fled more than the high-ranked helpers. We must note that, in Chapter 2, we only considered the helpers’ head-up displays in response to the aggression of the dominant male, whereas here we examined all the submissive behaviours performed by the helpers in response to the aggressive behaviours of the breeding pair. Body size is known to be a crucial factor in determining fighting ability in various animal species, and it is generally challenging for much smaller individuals to emerge victorious in such confrontations (Parker, 1974). It is known that, in dyadic contests within daffodil cichlids, the fish with a 5% or greater difference in body size is typically the winner during conflict, suggesting that smaller fish are unlikely to win the contests (Reddon et al., 2011). Indeed, smaller fish facing a much larger opponent have a lower chance of success, and the perceived value of such a chance may be minimal. Our findings seem to be in line with other studies in which subordinate males tend to exhibit greater overall submission when similar in size to the breeder male (Hamilton et al., 2005), as these closely matched fish are also likely to interact more frequently in general (Dey et al., 2013). Smaller and weaker animals are at higher risk of injury when attacked by larger and stronger animals (Lane & Briffa, 2017), which raises the potential costs of the interaction. Thus, the small helpers may have found it more suitable to flee from the aggression rather than staying and submitting while taking the risk of being attacked again.

In conclusion, we found that in daffodil cichlids, the aggression levels of the breeders were modulated by the manipulation of the environment in which our groups lived. Consequently, the behaviour of the helpers in our groups was influenced, with both submissive behaviours and fleeing being affected by aggression and varying depending on the experimental condition. However, the workload of our helpers was not affected by the aggression received nor by the environment. Indeed, we found that helpers of different ranks had alternative responses to the aggressive behaviour of the breeders, with the larger helpers performing more submissive behaviours, whereas the smaller ones were more prone to flee from aggression. Our findings help to shed light on the effect of the physical environment in modulating within group interactions of daffodil cichlids, an emerging model for the investigation of social behaviour.

Chapter 4: Dominance and aggression correlate with vasotocin neuronal phenotypes in a highly social fish

Tommaso Ruberto, William T. Swaney, Adam R. Reddon

4.1 ABSTRACT

Dominance hierarchies emerge when individuals of the same social group compete for resources. While social rank is associated with differences in behaviour and physiology, the mechanisms underlying hierarchical societies and their control mechanisms are not yet fully understood. Arginine vasotocin (AVT) is a nonapeptide hormone produced in the hypothalamus which plays a fundamental role in regulating dominance and social behaviours. However, the association between AVT neuronal phenotypes and the manifestation of social behaviour in fishes varies among species. In this study, we examined the agonistic behaviours of daffodil cichlids (*Neolamprologus pulcher*), an emerging model system for social behaviour, and compared them with the number and size of AVT immunoreactive neurons in the preoptic area of the brain. Subordinate fish had more parvocellular vasotocin neurons than dominant individuals, and dominants had overall larger cells than subordinates. Furthermore, the levels of aggression within their social groups were correlated with the number of parvocellular and magnocellular neurons, with males consistently displaying higher levels of aggression than the females. Aggressive behaviours in male dominants were not related to the AVT neuron size, but females showed a correlation between aggression and parvocellular neuron area. Finally, we did not find any correlation between AVT cells and submissive behaviours in the subordinates. Understanding the relationship between AVT and behaviour in daffodil cichlids can shed light on the evolution and regulation of social behaviour in fish, as well as provide insights into the neuroendocrine mechanisms underlying social behaviour in general.

Keywords: daffodil cichlids, agonism, social status, vasopressin, AVT, Neolamprologus pulcher

4.2 INTRODUCTION

Dominance hierarchies arise when there is competition among individuals of the same social group for access to resources such as food, mates, or shelter/habitats. The concept of dominance is based on repeated agonistic interactions between two individuals, resulting in a consistent outcome favouring one member of the pair and a yielding response from the other (Schjelderup-Ebbe, 1922; Drews, 1993). Dominant and more aggressive individuals at the top of the hierarchy typically retain priority access to resources (such as food and shelter), and usually have higher reproductive success than lower-ranking and more submissive individuals (Wingfield & Sapolsky, 2003; Sapolsky, 2005; Zhou et al., 2018). However, dominance is a relative measure and not an absolute property of individuals. Differences in behaviour and physiology are often associated with social ranks, and hormones are also known to vary between individuals of different social status (Sapolsky, 1982; Bartolomucci et al., 2001; Maruska et al., 2022), although their specific roles in determining or maintaining social status are not well understood in many species. Given the significant impact of dominance hierarchies on survival and reproductive success of a species, it is important to conduct integrative and comparative studies to investigate the mechanisms underlying hierarchical societies and their associated control mechanisms.

Nonapeptides are a highly conserved class of neuropeptides among vertebrates. These peptides have been identified as key molecules in various physiological processes, including cardiovascular function, osmoregulation, and stress (Banerjee et al., 2017). Furthermore, the nonapeptides play an important role in regulating social behaviours in animals (Bass & Groberb, 2001; Goodson & Bass, 2001; Goodson et al., 2003; Balment et al., 2006; Thompson et al., 2006; Godwin & Thompson, 2012), and they may also promote offensive aggressive behaviours (Ferris & Delville, 1994), social avoidance (Thompson & Walton, 2004), and aggressive responses to perceived threat (De Dreu et al., 2010). Arginine vasotocin (AVT), the non-mammalian homologue of the arginine vasopressin, is a nonapeptide which acts as both neurotransmitter and neuromodulator in the central nervous system of teleosts (Kulczykowska, 2008; Goodson & Thompson, 2010; Godwin &

Thompson, 2012), birds (Goossens et al., 1977) and amphibians (Moore & Lowry, 1998; Moore et al., 2005). AVT is mainly produced by neurons located in the preoptic area (POA) of the anterior hypothalamus. These neurons project to various areas, including the neurohypophysis, where AVT is released into the bloodstream to act peripherally (Godwin & Thompson, 2012), as well as to the ventral telencephalon, ventral thalamus, and mesencephalon (Saito et al., 2004; Huffman et al., 2012).

To regulate social behaviour, the integration of somatomotor expression with multiple coordinating influences is essential. These influences include sensory inputs from the environment, such as social stimuli and seasonal cues, as well as internal stimuli related to the endocrine state (Wilczynski, 1992). Additionally, a precise modulation of sensorimotor and physiological processes is necessary to generate an appropriate profile of complex, sex-typical, and species-specific behaviours. AVT has emerged as a key component in coordinating such complex behavioural expressions across a wide range of vertebrate species (Goodson & Bass, 2001). AVT expressing neurons can be categorized into different populations, including parvocellular, magnocellular and gigantocellular neurons, and throughout the course of vertebrate evolution, the locations of AVT neurons and fibres have demonstrated a remarkable degree of conservation (Moore & Lowry, 1998; Goodson & Bass, 2001). Even though the analysis of vertebrate AVT receptor gene sequences reveals a remarkable conservation of the core-ligand receptor interaction sites, it also exhibits variability in the intracellular components, consequently leading to diverse downstream effects (Cho et al., 2007). Moreover, in multiple vertebrate classes, divergence in social tactics has been associated with intra- or interspecific divergence in the distribution of AVT neural elements. Therefore, a comprehensive understanding of the nonapeptide-producing cells in the brain of model species, specifically in teleosts, may help unravelling the impact of AVT on social behaviour and examine hypotheses pertaining to brain evolution and function (Godwin & Thompson, 2012; Thompson & Walton, 2013).

Many studies performed in several fish species have investigated the link between social behaviour and the role of nonapeptides on regulating aggressive and submissive behaviours (Almeida et al., 2012; Kleszczyńska et al., 2012; Reddon et al., 2012; Lema et al., 2015; Perrone & Silva, 2018), and it is now widely known that arginine vasotocin (AVT) plays a major role in regulating social interactions, such as aggression and territoriality (Dewan & Tricas, 2011; Kulczykowska & Kleszczyńska, 2014; Almeida & Oliveira, 2015; Teles et al., 2016; Loveland & Fernald, 2017). Yet, the correlation between the organization of the AVT neurons (that is, AVT neuronal phenotypes) in the brain and the expression of status-dependent social behaviour in fish is not consistent across species. For example, in the African cichlid *Astatotilapia burtoni* levels of AVT within gigantocellular neurons correlated with the expression of aggressive and reproductive behaviours of dominant individuals, and AVT in the parvocellular neurons was strongly correlated with fleeing behaviour in subordinate individuals (Greenwood et al., 2008). Similarly, in other teleost species, a greater quantity or size of magnocellular or gigantocellular AVT immunoreactive neurons have been linked with territorial behaviours and/or social dominance, whereas a larger number of parvocellular AVT neurons have been linked with submission (e.g. zebrafish, *Danio rerio* (Larson et al., 2006) and butterfly fishes (Dewan et al., 2008; Dewan et al., 2011; Dewan & Tricas, 2011)). On the other hand, pupfish (*Cyprinodon nevadensis*) display varying levels of aggressive behaviour between two geographically separated populations. In the more aggressive population, males tend to have smaller parvocellular AVT neurons compared to the less aggressive population (Lema, 2006). It is worth mentioning that so far most of the studies investigating the relationship between nonapeptides and social behaviours have examined fish species with relatively simple social systems and or sex specific dominance hierarchies. Thus, the considerable variation among species, as well as the different methods used in various studies, pose a challenge to identifying general patterns.

The daffodil cichlid (*Neolamprologus pulcher*) is a cooperatively breeding cichlid fish that serves as an emerging model system in the field of integrative biology of social behaviour. This species is native to the rocky littoral zone in Lake Tanganyika, East Africa (Taborsky & Limberger, 1981; Balshine et al., 1998) and exhibits a sophisticated dominance-based social system characterized by frequent social interactions and specialized behaviours and signals (Wong & Balshine, 2011a). Daffodil cichlids live and breed in social groups consisting of a dominant breeding pair and 1-20 non-breeding adult subordinates of both sexes who assist the dominant pair in clearing the territory, defending against predators and competitors, and caring for the dominants' offspring (Taborsky, 1984; Taborsky, 1985; Balshine et al., 2001; Heg et al., 2005; Desjardins et al., 2008). The small size, fast growth, and adaptability to aquarium conditions of this species facilitate the formation of naturalistic social groups and the performance of their full suite of social behaviours in captivity. Moreover, due to its exceptional social nature and amenability to controlled laboratory experiments, daffodil cichlids are an attractive study system for unravelling the role of nonapeptide hormones in regulating social behaviour.

Among daffodil cichlids groups, the maintenance of social rank in the dominance hierarchy is a continuous process that involves the use of different types of behaviour, such as aggressive, submissive, and affiliative interactions (Wong & Balshine, 2011a). Like other group-living species, vacancies in the dominance hierarchy can arise due to natural deaths or predation events. When these vacancies occur in high-ranking breeding positions, individuals from both within and outside the group may compete for the newly available position (Balshine et al., 1998; Stiver et al., 2004; Fitzpatrick et al., 2008). However, the contextual link between behaviour and status (that is, aggressive when dominant and submissive when subordinate) and how that may be controlled by the brain and the hormones is still a matter of debate. For example, previous studies have shown that AVT may regulate the behaviour of the daffodil cichlids, including aggression, courtship, and parental care (Reddon et al., 2015; Reddon et al., 2017), suggesting that AVT may have a crucial role in determining the social status of individuals in this species. In fact, it was observed that dominant daffodil cichlids had a higher expression of the AVT gene as compared to subordinates (Aubin-Horth et al., 2007), although subordinates seem to have higher concentrations of bioavailable AVT in their brains compared to dominants (Reddon et al., 2015). Thus, the exact role that AVT plays in mediating agonistic behaviour, and the correlation between AVT neuronal phenotypes and the expression of such behaviours is still not fully understood.

To this end, we sought to identify any possible correlation between social status, agonistic behaviour and AVT neuronal phenotypes in daffodil cichlids. In particular, we examined the agonistic behaviours of daffodil cichlids' social groups reared under naturalistic conditions, and we correlated the behavioural data and the sex/rank of the group members with the individual quantity and size of AVT cell types in each cell population. First, we anticipated differences in the number and/or size of AVT-containing cells between dominant and subordinate individuals. In particular, based on observations made in other social fish species (Larson et al., 2006; Dewan et al., 2008; Greenwood et al., 2008; Dewan et al., 2011; Dewan & Tricas, 2011), we expected dominants to have more/larger magnocellular and/or gigantocellular neurons than subordinates, but potentially the same number or even fewer parvocellular neurons. We also predicted a correlation between agonistic behaviours of daffodil cichlids and AVT neuronal phenotypes: we predicted that, among dominant individuals, more aggressive individuals also would also have higher numbers and/or larger AVT cells, particularly in the magnocellular and gigantocellular populations; likewise, we predicted a positive relationship between submissive behaviours of the subordinate individuals and the number and/or size of the parvocellular neurons.

4.3 MATERIALS AND METHODS

4.3.1 Study animals

The research subjects were laboratory reared daffodil cichlids (*Neolamprologus pulcher*) descended from fish captured on the southern shore of Lake Tanganyika, Africa. Prior to the experiment, all fish were kept in mixed-sex groups, with a density of approximately 50 fish per aquarium (105 × 43 x 40cm, 180-litre). Each stock aquarium was equipped with a heater, a thermometer, two powered filters, an air stone, and 3 cm of fine coral sand. Temperature was maintained at 27 ± 1°C on a 12:12 h light:dark day cycle, with 30 min of gradual transition from light to dark to simulate sunrise and sunset. The aquaria were regularly checked for water quality parameters and weekly cleanings were performed. Fish were fed daily with a mix of dried flake and pellet cichlid food (Tetra Werke, Germany).

4.3.2 Focal groups

Focal subjects were moved into 90L experimental aquaria (53 × 43 x 38 cm) to form ten social groups. Each experimental aquarium was equipped with two foam filters, a heater, and a thermometer, along with 3 cm of fine coral sand. Furthermore, each aquarium was furnished with 4 terracotta caves, which could be used by the fish as breeding substrate as well as shelter, and two floating translucent green PET bottles attached to the aquaria, which provided additional refuge for the fish. All fish were kept under the same husbandry regime described above. The social groups included two dominant breeder fish (male and female) and 6-7 subordinates. Sex was determined by examination of the genital papillae only in the dominant pair and then confirmed post-mortem. Daffodil cichlids usually reach sexual maturity around an age of 1 year (Taborsky, 1985), and in our experiments, subordinates were often too young to be sexed with certainty, even after post-mortem analyses. To avoid any possible misattribution, we decided to not to attempt to sex the subordinates in this study. The sizes of the breeding fish and of the two largest subordinates (“subordinate 1” and “subordinate 2”) were recorded (mean ± SE standard length, measured from the tip of the snout to the end of the caudal peduncle: dominant male = 5.29 ± 0.13 cm; dominant female = 4.86 ± 0.14 cm; subordinate 1 = 3.71 ± 0.08 cm, subordinate 2 = 3.48 ± 0.08 cm). To form the social groups, we introduced subordinate individuals into the experimental tanks 24 hours before the breeding pair. Groups were housed together for at least one month prior to observation and had successfully spawned at least once. We carefully observed the new groups and checked for excess overt aggression or the social rejection of any group members. At the time of observation, all groups contained fry (<1cm standard length).

4.3.3 Experimental procedures

Behavioural scoring was performed manually during the experiments by a trained observer (T.R.). We observed each group for four 30-minute periods over the course of a week, resulting in a total of 120 minutes of observation per group. The observations were performed between 10h and 18h and only one observation was done per day. The observations were taken by a stationary observer seated approximately 1.5m from the front of the aquarium. A 10-minute habituation period prior to the onset of coding allowed the fish to acclimate to the presence of the observer. During the behavioural observations, we recorded all interactions between each of the four focal group members (dominant male, dominant female, subordinate 1, subordinate 2) and behaviours directed by them towards the smaller subordinates. The five different aggressive behaviours recorded were: chases, rams, bites, head down displays, and frontal displays. The submissive behaviours recorded were the head-up displays and tail quiver displays (Ruberto et al., 2020; Manara et al., 2023). For a detailed description of these behaviours, see (Reddon et al., 2015).

After the last observation, we removed the four focal fish from the aquaria and euthanised them with an overdose of the anaesthetic tricaine methane sulfonate (MS222, 300mg/L, Sigma-Aldrich, Dorset, UK) dissolved in tank water and buffered to pH 7.4. Briefly, subjects were immersed in the MS222 for up to 10 minutes and observed until the absence of opercular movement. Fish were then decapitated, the brain was dissected and placed in a solution of 4% paraformaldehyde in 0.1M Phosphate buffered saline (1x PBS), then stored overnight at 4°C. The following day, the brains were cryoprotected by placing them in a 30% sucrose solution and storing them overnight at 4°C. Following cryoprotection, brains were placed in moulds containing Optimal Cutting Temperature (OCT) compound and then rapidly snap-frozen in dry ice-chilled hexane. Whole brains were sectioned coronally into slices 30μm thick using a Leica CM3050s Cryostat and thaw mounted on Superfrost Plus Gold slides (Fisher Scientific, Loughborough, UK) in two parallel series. Slides were subsequentially stored at -20°C until ready to be processed for immunohistochemistry.

4.3.4 Immunohistochemistry process and antibody validation

For our experiments, we used the rabbit α-Arginine Vasopressin (α-AVP, catalogue n. 20069, ImmunoStar, Hudson, WI, USA) primary antibody (Marsh et al., 2006; Subhedar et al., 2008). To validate antibody binding specificity in daffodil cichlid, we carried out a pre-incubation staining procedure using the α-AVP on samples of brain tissue of daffodil cichlids from our stock tanks. We pre-incubated the α-AVP (1:1000) with a surplus of either 50μMol of pure Arginine Vasotocin (AVT) protein (Bachem, St Helens, UK), 47μMol of pure Isotocin (IT) protein (Bachem, St Helens, UK), a mix of both pure proteins, and a control condition without protein pre-incubation. If the α-AVP would reliably bind to the AVT, the staining should be prevented by the AVT protein pre-incubation, since the antibody would be fully bound to that protein during pre-incubation. On the other hand, if the staining would be blocked by the IT protein, this would indicate that the primary antibody has cross-reactivity for this closely related nonapeptide. Finally, if staining was not inhibited by pre-incubation with either protein, then this would demonstrate that the α-AVP does not bind to either nonapeptide in this species.

Sections were thawed and air-dried for 15 minutes before being outlined with a hydrophobic pen to avoid liquid spills during incubations, and subsequentially washed in 1x PBS three times for five minutes. Sections were incubated in 350ml of 2% normal goat serum (Vector Laboratories, Peterborough, UK) diluted in 1x PBST (1x PBS +0.1% Triton X-100) blocking buffer for 60 minutes in a moist chamber to block nonspecific antibody binding. After this step, the sections were incubated according to the validating condition (that is, α-AVP with AVT, α-AVP with IT, α-AVP with AVT+IT, α-AVP with no protein) in blocking buffer and stored at 4°C overnight in a moist chamber.

The following day, the slides were washed three times in 1x PBS for 5 minutes and then incubated in the dark with Goat anti-Rabbit IgG H&L preabsorbed secondary antibody (1:1000) (Alexa Fluor™ 555, catalogue n. ab150078, Abcam, Cambridge, UK) in blocking buffer for 1 hour at room temperature in a moist chamber. The slides were then washed three times in 1x PBS and once in 1x PBST for 5 minutes in the dark. After the last wash, slides were briefly dipped in de-ionized water to remove excess salt, and three/four drops of Fluoroshield + DAPI mounting medium (Abcam, Cambridge, UK) were added before carefully mounting coverslips onto the slides. Finally, slides were sealed with clear nail varnish to prevent drying or moving of the sections under the microscope, and then stored in a sealed container at 4°C to prevent photobleaching until the imaging process. Findings from pre-incubation staining revealed that α-AVP binding was blocked by pre-incubation with AVT but not by IT, confirming the specificity of α-AVP in daffodil cichlids. Consequently, we performed all the immunohistochemistry processes for our brain samples using the validated α-AVP, using the method described above but omitting the pre-incubation with AVT/IT proteins.

4.3.5 Imaging and measuring cells

The imaging process was performed with a Leica LMD6 fluorescent microscope using 20x magnification. Individual brain sections were imaged using RHO (absorption peak = 541-

551nm) and DAPI (absorption peak =340-380nm) fluorescence filters. Neurons were discriminated by manually identifying the cell type based on soma size and localization: parvocellular cells (Fig. 1) were identified as densely-packed smaller neurons located in the anterior and ventral region of the preoptic area (POA) of the brain, magnocellular neurons (Fig. 2) as mid-sized neurons located in the posterior and dorsal region throughout the POA, and gigantocellular neurons (Fig. 3) as large neurons in the posterior and dorsal region of the POA. All the AVT cell types in the POA were counted independently and quantified manually during the imaging process, and the final cell counts were divided by the number of sections to achieve a per section cell count index for each cell type in each fish.

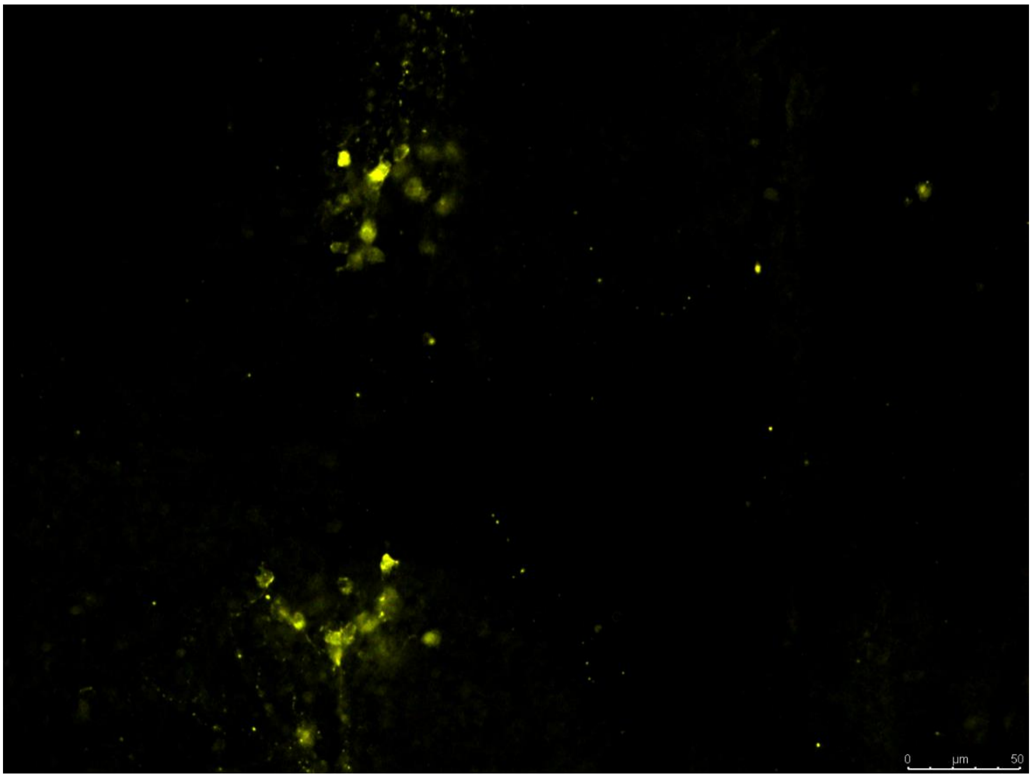

Figure 1: Parvocellular neurons imaged at 20x magnification of the dominant female daffodil cichlid from the 4th experimental group.

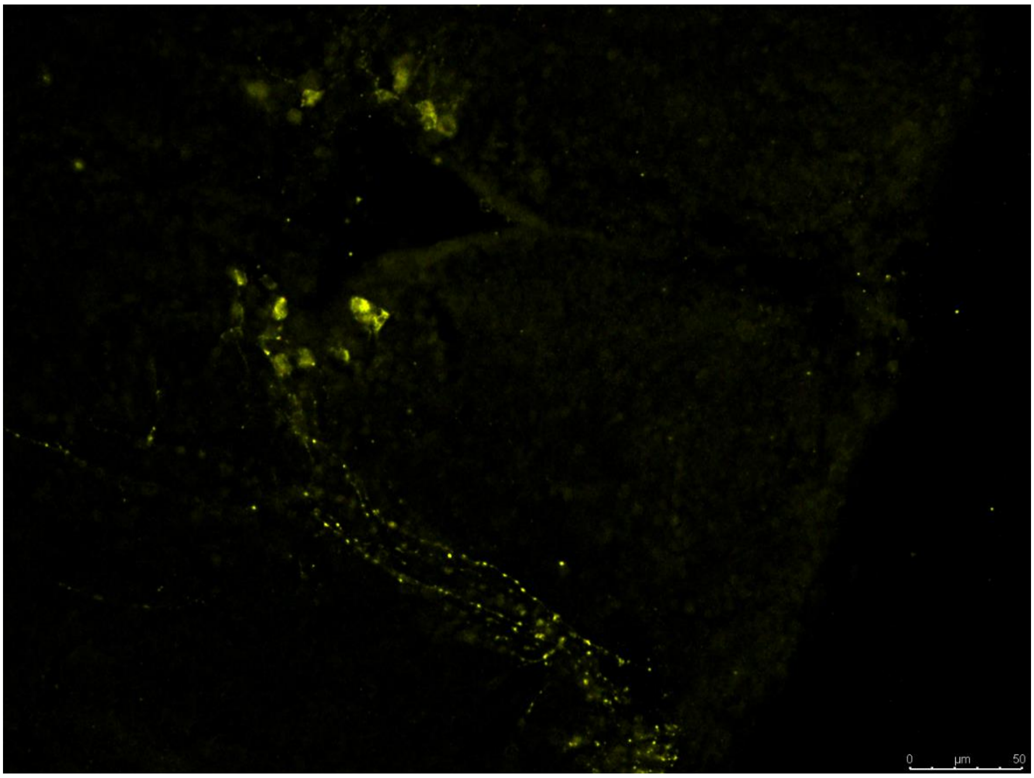

Figure 2: Magnocellular neurons imaged at 20x magnification of the dominant female daffodil cichlid from the 4th experimental group.

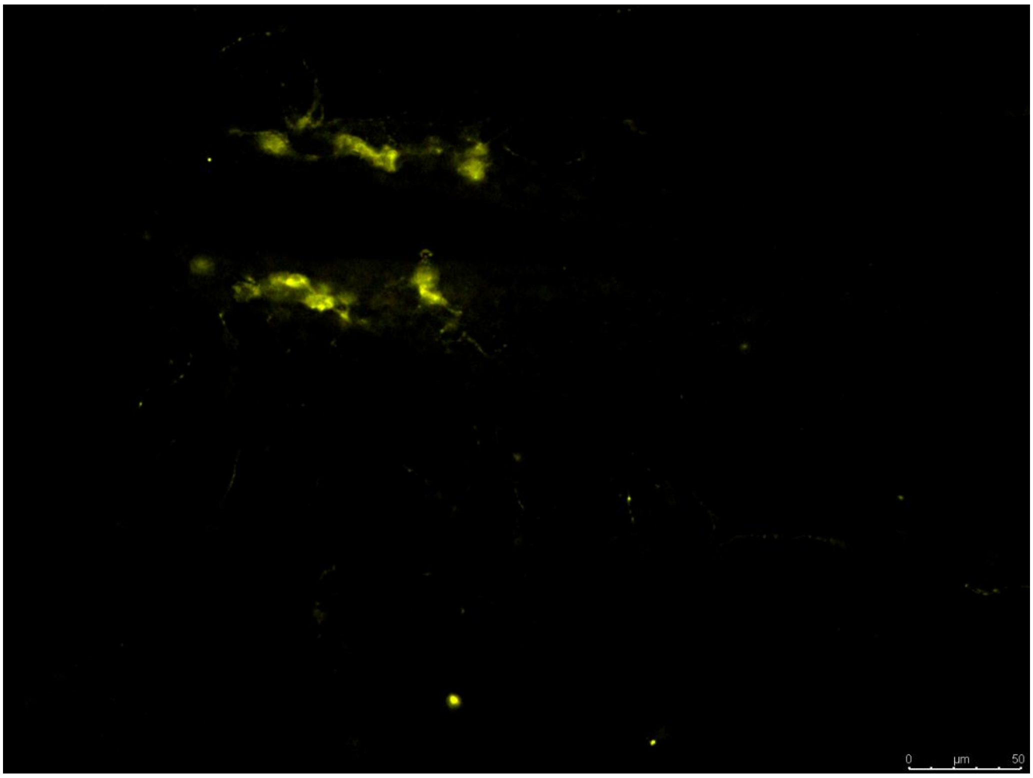

Figure 3: Gigantocellular neurons imaged at 20x magnification of the dominant female daffodil cichlid from the 4th experimental group.

Pictures of the brain sections were also taken during the imaging process for measuring the cell areas. To this end, we analysed the pictures of the brain samples with ImageJ. For each brain sample, we randomly selected up to 10 neurons for each cell type. When less than 10 neurons for cell type were found in the sample, we selected all the neurons. Neurons were then manually measured by carefully placing a contour around them and then the area was measured through the “Measure” feature of the ImageJ software. Finally, we averaged the measures and obtained a mean value for each cell type in each fish. Due to tissue loss during the sectioning process, one female dominant and one subordinate brain sample were not quantified, while for another subordinate we gathered data only for the gigantocellular cells.

4.3.6 Statistical analysis

To test whether the behaviours of our focal fish were correlated to the different AVT neuronal phenotypes, we compared the behavioural data that we gathered with the cell measurements from the brain samples, focusing on the number and size of the AVT immunoreactive cells.

First, we conducted a preliminary Linear Mixed Models (LMMs) fit to a Gaussian distribution to test whether body length predicted the cell counts or the cell areas in dominant females, dominant males, or subordinates. As these preliminary models showed no effects of body length on cell counts or cell areas within fish ranks, we did not include this as a predictor in the following models. Then, we conducted LMMs fit to a Gaussian distribution to test possible differences between cell counts and cell areas depending on the rank of the focal fish. We coded either cell count index or average area as a dependent variable and rank (dominant or subordinate) as predictors. Group identity was included as a random factor.

Next, for each cell type, we conducted LMMs fit to a Gaussian distribution, with either aggressive behaviours (for the dominants) or submissive behaviours (for the subordinate) as response variables, and either cell count index or average area as fixed factors. For the analyses on the dominants, we also included sex and body length as fixed factors, and non-significant terms were sequentially removed to reach minimal models. Group identity was again included as a random factor. For the dominants, we compared the aggression levels with the counts and the areas of each cell type found in their brain samples, as dominant individuals seldom produced submissive behaviours, while for the subordinates who did not show aggression towards the dominants, we compared the submissive behaviours with the counts and the areas of the above-mentioned cell types. For all our models, we square-root transformed our behavioural data to account for right skew, and fixed effects were tested with likelihood ratio tests.

All our analyses were performed using R version 4.2.3 (R-Core-Team, 2023) and R Studio (2022.07.02, Build 576), and the packages “lme4” (Bates et al., 2015) to fit our LMMs, and “lmerTest” (Kuznetsova et al., 2017) and “car” (Fox & Weisberg, 2018) to evaluate the main effects and interactions in each model. Models were assessed using the “performance” package (Lüdecke et al., 2021) to check that model assumptions were met.

4.3.7 Ethical note

Animal housing, handling, and study protocols were approved by the Liverpool John Moores Animal Welfare and Ethics Steering Group (approval number: AR_TR/2018-4) and adhered to the guidelines of the Animal Behaviour Society and the Association for the Study of Animal Behaviour. All fish were closely monitored for social exclusion or signs of injury. All observations were drawn from stable social groups showing typical levels of agonism for daffodil cichlids (Balshine et al., 2017).

4.4 RESULTS

Social rank influenced the number of parvocellular neurons in the POA ($chi^2 = 12.194$, $p < 0.001$), but not the number of magnocellular neurons ($chi^2 = 2.375$, $p = 0.123$) or of the gigantocellular neurons ($chi^2 = 2.141$, $p = 0.143$; Fig. 4A). In particular, the dominants had a higher cell count index for the parvocellular cells than the subordinates. The rank of the fish was found to influence the average cell area of the parvocellular neurons ($chi^2 = 6.669$, $p < 0.001$), of the magnocellular cells ($chi^2 = 16.796$, $p < 0.001$) and of the gigantocellular cells ($chi^2 = 24.524$, $p < 0.001$; Fig. 4B). In particular, the dominants had a higher average cell area for all the cell types the subordinates.

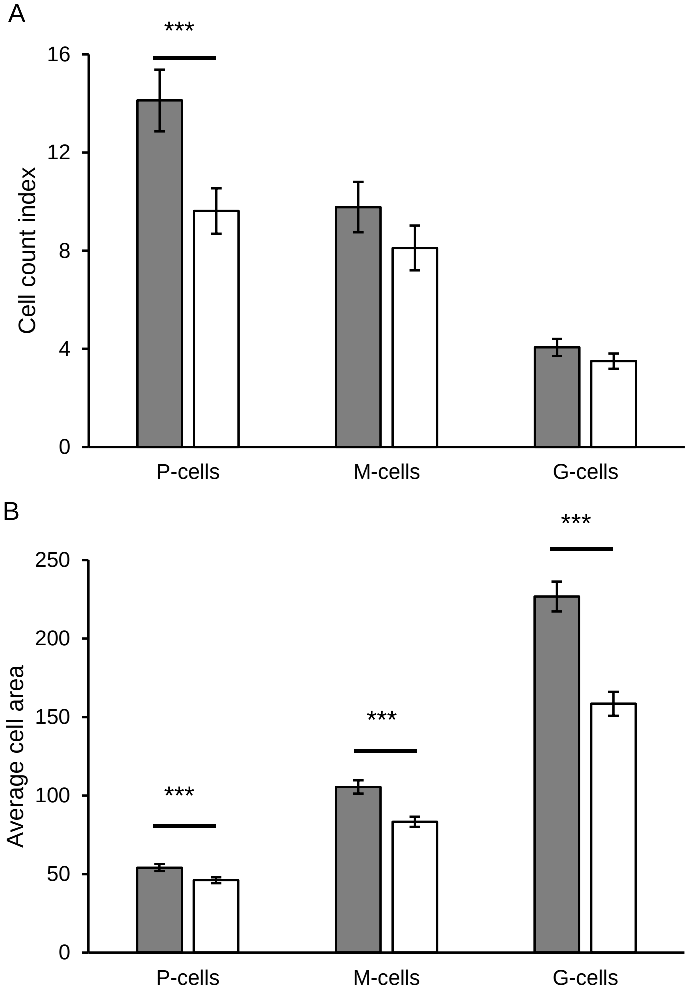

*Figure 4. The cell counts per section (A), and the average cell areas (B) of dominants (grey bars) and subordinates (white bars) in daffodil cichlids for parvocellular AVT neurons (Parvo), magnocellular AVT neurons (Magno), and gigantocellular AVT neurons (Giganto). The * indicates a p<0.05, ** indicates a p<0.005, *** indicates a p<0.001.*

For the analyses on the cell counts, the body length and the interaction between sex and cell counts were removed from all the models as they were not significant in any of the models. In dominants, aggressive behaviours were positively correlated with the number of parvocellular cells ($chi^2 = 5.214$, $p = 0.022$; Fig. 5A) and magnocellular cells ($chi^2 = 6.813$, $p = 0.009$; Fig 5B). However, we did not find a significant relationship between the number of gigantocellular cells and aggressive behaviour ($chi^2 = 7.000$, $p = 0.403$; Fig. 5C). There was a significant effect of sex on aggression in the parvocellular ($chi^2 = 9.788$, $p = 0.002$), magnocellular ($chi^2 = 9.609$, $p = 0.002$), and gigantocellular cells ($chi^2 = 8.642$, $p = 0.003$), with males systematically displaying higher levels of aggression than the females.

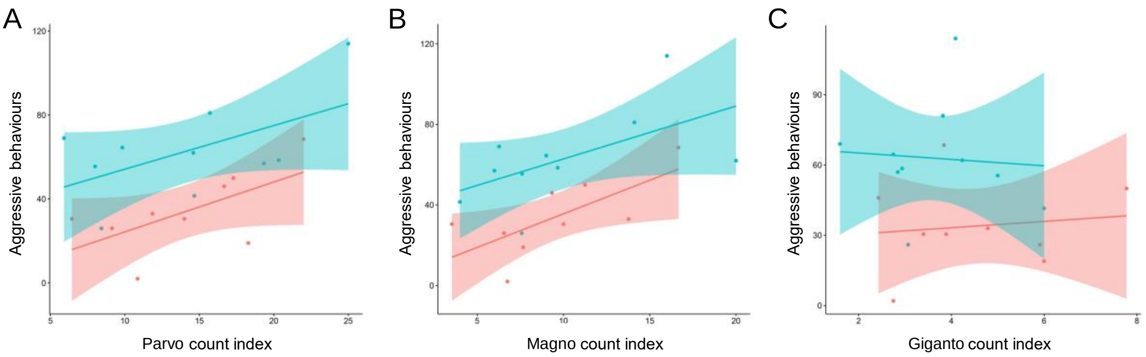

Figure 5. The correlation between cell counts indexes and aggressive behaviours of female (light red) and male (light green) daffodil cichlids for parvocellular AVT neurons (Parvo) (A), magnocellular AVT neurons (Magno) (B), and gigantocellular AVT neurons (Giganto) (C). Linear best fit line with 95% confidence intervals (shading) are displayed.

Similar to the analyses on the cell counts, for the cell areas the body length was dropped in all the models as it was never significant. However, the analyses performed on the cell areas showed that there was an interaction between sex and cell areas for all the cell types (parvocellular cells: $chi^2 = 7.841$, $p = 0.005$, Fig. 6A; magnocellular cells: $chi^2 = 7.303$, $p = 0.007$, Fig. 6B; gigantocellular cells: $chi^2 = 4.497$, $p = 0.034$, Fig. 6C). In particular, aggressive behaviours of the dominants were affected by sex (parvocellular cells: $chi^2 = 6.656$, $p = 0.010$; magnocellular cells: $chi^2 = 6.902$, $p = 0.009$; gigantocellular cells: $chi^2 = 7.024$, $p = 0.008$), but not by the cell areas (parvocellular neurons: $chi^2 = 0.155$, $p = 0.694$; magnocellular cells: $chi^2 = 0.323$, $p = 0.570$; gigantocellular cells: $chi^2 = 0.014$, $p = 0.905$).

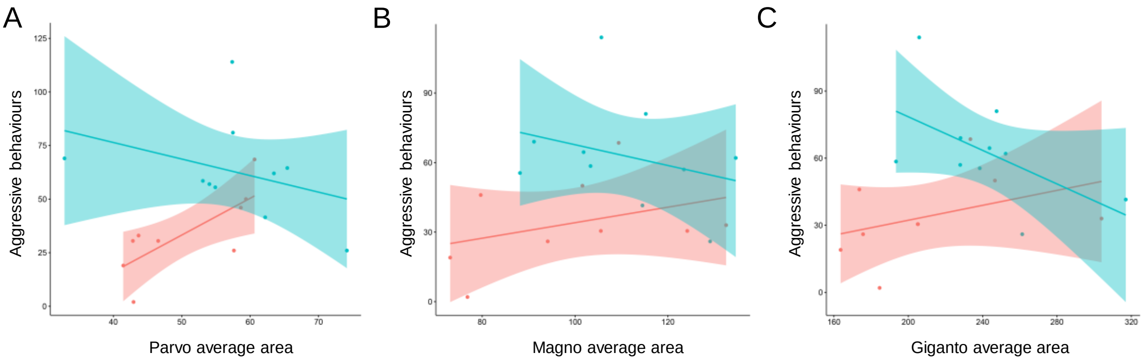

Figure 6. The correlation between average cell areas and aggressive behaviours of female (light red) and male (light green) daffodil cichlids for parvocellular AVT neurons (Parvo) (A), magnocellular AVT neurons (Magno) (B), and gigantocellular AVT neurons (Giganto) (C). Linear best fit line with 95% confidence intervals (shading) are displayed.

Finally, the analyses performed on the subordinates showed that submissive behaviours were not correlated neither with the cell counts (parvocellular cells: chi^2 = 1.292, p = 0.196; magnocellular cells: chi^2 = 0.646, p = 0.289; gigantocellular cells: chi^2 = 0.165, p = 0.679; Fig. 7) nor with the cell areas (parvocellular cells: chi^2 = 0.109, p = 0.76; magnocellular cells: chi^2 = 0.121, p = 0.736; gigantocellular cells : chi^2 = 0.178, p = 0.621; Fig. 8).

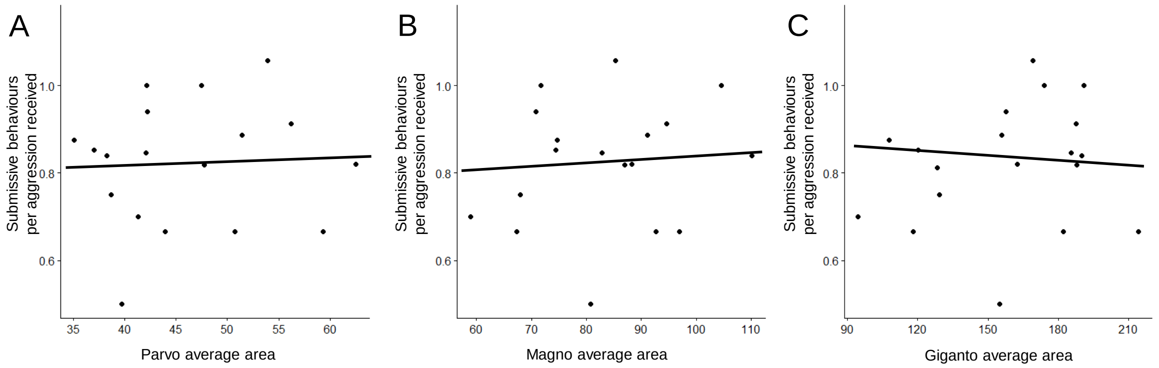

Figure 7. The correlation between cell counts indexes and submissive behaviours per aggression received in daffodil cichlid subordinates for parvocellular AVT neurons (Parvo) (A), magnocellular AVT neurons (Magno) (B), and gigantocellular AVT neurons (Giganto) (C). Linear best fit lines are displayed.

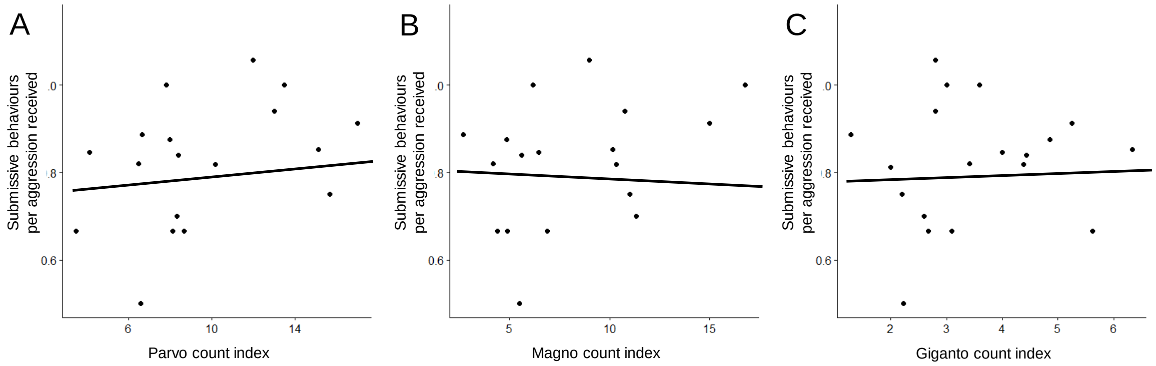

Figure 8. The correlation between average cell areas and submissive behaviours per aggression received in daffodil cichlid subordinates for parvocellular AVT neurons (Parvo) (A), magnocellular AVT neurons (Magno) (B), and gigantocellular AVT neurons (Giganto) (C). Linear best fit lines are displayed.

4.5 DISCUSSION

In this study, we evaluated the status-dependent differences in AVT neuronal phenotypes as a pathway to control status-specific behavioural responses in the cooperatively breeding daffodil cichlid. We sought to understand how the AVT neuronal phenotypes would differ between ranks and sexes by analysing the correlations between behaviours and the expression of AVT neurons in the POA. We expected that subordinates would have fewer and smaller cells compared to dominants, especially among the magno- and gigantocellular neuron populations. Additionally, we predicted that more aggressive individuals would have a greater number and/or larger AVT magno- and gigantocellular neurons. We also anticipated that submissive behaviours in subordinate individuals would have a positive correlation with parvocellular neurons. We found that the dominants had more parvocellular neurons and larger AVT cells overall compared to subordinates. Aggressive behaviours in the dominants were positively correlated with the parvo- and magnocellular neuron counts, and sex influenced the behaviours of the fish, with males being more aggressive than females. However, we did not find any relationships between aggressive behaviours and cell areas. With respect to the subordinates, we found no relationships between submissive behaviours and AVT neurons, for either cell counts or the cell areas.

Previous reports in daffodil cichlids showed that dominant individuals had higher brain AVT gene expression compared to subordinates (Aubin-Horth et al., 2007). Although AVT cell count or size data may differ with gene expression data (as for example in the fish species *Salaria pavo* (Grober et al., 2002)), our results seem to be in line with previous findings in daffodil cichlids (Aubin-Horth et al., 2007), as we found that the dominant individuals had more parvocellular cells and overall larger AVT cells compared to the subordinates. It is plausible that greater AVT gene expression could be reflected in an increased production of AVT, and that a higher quantity or larger size of AVT cells may suggest a greater capacity for storage (Ota et al., 1999). However, these results contrast with a previous study, in which subordinates of daffodil cichlids had higher levels of free bioactive AVT in their brains than dominants (Reddon et al., 2015), although we did find that subordinates had less parvocellular AVT cells than dominants. It is important to note that measures of gene expression may not always directly correlate with final concentrations of the bioactive nonapeptide due to the various steps involved between mRNA production and the eventual end products. Moreover, variations in gene expression typically correspond to differences in AVT production, while the amount of available peptides may signify differences in storage. For instance, dominant and subordinate fish may exhibit discrepancies in the extent to which AVT could be released to the periphery versus being retained in the brain (Almeida et al., 2012; Reddon et al., 2015).

Although AVT has been linked to the modulation of agonistic behaviours in several teleosts (Backström & Winberg, 2017), the role of AVT in controlling behaviour is likely to differ among different species and social contexts (Teles et al., 2016). For example, in zebrafish (*Danio rerio*) dominant individuals have a greater number of magnocellular AVT neurons in the POA, and these cells were also larger compared to subordinates. On the other hand, in the parvo-cellular POA, subordinate zebrafish have larger and more numerous AVT cells than dominant individuals (Larson et al., 2006). Conversely, in Mozambique tilapia (*Oreochromis mossambicus*), subordinate males have larger magnocellular AVT cells compared to territorial males and have a higher number of gigantocellular AVT cells, even though the number of parvo- and magnocellular AVT cells do not differ between the two male types (Almeida & Oliveira, 2015). In our experiments on daffodil cichlids, we found a positive correlation between aggressive behaviours and the number of parvo- and magnocellular AVT cells in both the female and the male dominant fish, and but not between aggression and the area of the AVT neurons. Moreover, a similar pattern of magnocellular activation after engaging in aggressive behaviours was found in dominant males of *A. burtoni* (Loveland & Fernald, 2017), which is also a pattern consistently found in other animal models (Gouzènes et al., 1998). Interestingly though, the gigantocellular AVT neurons were not correlated in any way with the aggressive behaviours displayed by the dominants. In *A. burtoni*, an African cichlid species with a lek mating system, territorial males exhibit higher expression of AVT mRNA in the gigantocellular neurons than non-territorial males, and AVT mRNA levels are correlated with aggressive behaviour (Greenwood et al., 2008).

Daffodil cichlids are a highly social vertebrate species with a complex and intricate hierarchy, in which both dominant individuals are compelled to aggressively defend their territory and display dominance to subordinate individuals, with males showing in general more aggression than females (Balshine et al., 1998). It may not be surprising that, in our experiments, different levels of aggression and AVT cells were found in both sexes, although it is also known that dominant females are similar to dominant males in terms of agonistic behaviours and AVT expression in the brain (Aubin-Horth et al., 2007). With respect to the subordinates, our findings showed an absence of correlations between AVT cell measures and the expression of submissive behaviours. Although these results are consistent with previous studies on this fish species (Reddon et al., 2015), in other fish species, such as *A. burtoni*, there is a strong correlation between submissive behaviours and higher AVT mRNA levels in the parvocellular POA among subordinates (Greenwood et al., 2008). Furthermore, along with the involvement in the production of aggressive behaviours, AVT seems to play a role in regulating the social status and ascension along the dominance hierarchy of social fishes (Semsar et al., 2001; Huffman et al., 2015). In daffodil cichlids, subordinate individuals must inhibit aggressive behaviours and show submission towards those ranked above them, and at the same time they must behave in the opposite way towards those below them in the hierarchy (Taborsky, 1985; Wong & Balshine, 2011b; Wong & Balshine, 2011a). It has been reported that isotocin, the teleost homologue oxytocin, seems to be involved in the production of submissive behaviours in daffodil cichlids (Reddon et al., 2012; Hellmann et al., 2015). It also is plausible that isotocin circuits are fundamental to the regulation of status and behaviour of subordinate daffodil cichlids.

In conclusion, we sought to elucidate the role of AVT in the expression of agonistic behaviours by analysing the different AVT neuronal phenotypes in the daffodil cichlid, an emerging model system for studying the proximate and functional aspects of social behaviour. To the best of our knowledge, this is the first study in which agonistic behaviours have been shown to be associated with different AVT neuronal phenotypes in this species, significantly enhancing our comprehension of the AVT system and its correlation with social status and behaviour in a cooperatively breeding vertebrate. Our findings show that in this species AVT is closely connected to the individual status in the hierarchy and to aggression, but not to submission. Our results will help unravelling the correlation between nonapeptides and behaviour and will also have broader implications for the comprehension of the neural control of social status and aggression in the animal kingdom, suggesting new perspectives into the neuroendocrine mechanisms that are involved in the development and regulation of social behaviour.

Chapter 5: Arginine-vasotocin involvement in the establishment of daffodil cichlid social hierarchies

Tommaso Ruberto, William T. Swaney, Adam R. Reddon

5.1 ABSTRACT

Agonistic behaviours play a vital role in the establishment of social hierarchies among animals. The ability to recognize one's own social rank, as well as the ranks of others, is crucial for group-living animals to prevent ongoing and costly conflicts. Yet, the behavioural and neurological mechanisms underlying the establishment of dominance hierarchies in social animals are still poorly understood. The hypothalamic neuropeptide arginine-vasotocin (AVT) is a key modulator of social behaviour and it is thought to play a role in the expression of aggressive behaviours and social position. To better understand the role of AVT on dominance establishment, we performed behavioural experiments in daffodil cichlids (*Neolamprologus pulcher*), a cooperatively breeding fish with a sophisticated dominance-based social system in which a pair of naïve fish were allowed to interact. Dominant–subordinate relationships were quickly established, with the larger fish of the pair becoming dominant and the smaller one subordinate. To evaluate the role of AVT during the establishment of the dominance hierarchy, fish brain tissue was processed at the end of the behavioural tests using double-labelling immunohistochemistry with an AVT antibody and a marker for recent neuronal activity. We did not find any difference AVT activated cells between dominant and subordinate fish, and the correlations between aggressive behaviours and AVT activated cells were not significant. Despite the non-significant results, the design of our experiments will help future investigations involving integrative and multidisciplinary approaches involving immunohistochemistry methodologies and behavioural observations.

Keywords: Neolamprologus pulcher, agonism, dominance, social hierarchies, AVT, pS6

5.2 INTRODUCTION

Agonistic behaviours, a set of behaviours that encompass social interactions involving conflicts between individuals of the same species, have played a significant role in shaping social dynamics throughout evolutionary history (Lorenz, 1966; King, 1973). Among social species, conflicts may arise due to competition for various resources such as territory, food, mates, breeding sites, and more, and they are resolved through one individual obtaining and maintaining control over the resource (dominant) while the other relinquishes it (subordinate). Although the specific behavioural traits exhibited during these contests may vary greatly among species, agonistic encounters consistently progress through three distinct phases: evaluation, contest, and post-resolution, with overt aggression typically occurring during the contest phase (Nelson, 2006; Summers & Winberg, 2006). This stable pattern of behaviour is the outcome of a complex evaluation process among competitors, enabling them to determine whether to escalate the conflict or concede (Maynard Smith & Parker, 1976). Consequently, during the post-resolution phase, a clear asymmetry in behaviour based on status is observed among the contenders, and this asymmetry relies on neuroendocrine mechanisms that regulate the emergence of either dominance or subordination.

Distinctive neuroendocrine mechanisms operating within the highly conserved vertebrate social brain network (SBN) govern the behavioural asymmetries between dominants and subordinates (Newman, 1999; Goodson, 2005; O'Connell & Hofmann, 2011; Kelly, 2022). The spatio-temporal activity patterns of the SBN, crucial for the emergence of status-dependent behaviours and the maintenance of stable hierarchies, are shaped by multiple neuromodulators acting through both fast wired circuits and slow diffusive pathways (Newman, 1999; Goodson & Kabelik, 2009; O'Connell & Hofmann, 2011). To investigate the short-term activation of the SBN, a commonly employed approach is to examine the expression of immediate early genes (IEGs) within its nodes through tightly controlled social behavioural experiments (Goodson et al., 2005; Fischer et al., 2018; Kabelik et al., 2018;

Williamson et al., 2019; Friesen et al., 2022; Ghahramani et al., 2022). IEGs are genes that undergo rapid transcription when cells respond to various cellular stimuli (Thomas & Brooks-Kayal, 2013; Barbosa & Silva, 2018), for example, neuronal activity after a learning process or a behavioural-state response (Long & Salbaum, 1998; Thomas & Brooks-Kayal, 2013; Barbosa & Silva, 2018). Noteworthy, the expression of these transcription factors after stimulation does not require neither protein synthesis nor translation (Hawk & Abel, 2017). IEGs play a vital role in synaptic plasticity and synaptogenesis, and many of these genes function as transcription factors and DNA-binding proteins, allowing them to trigger specific signalling pathways (Barbosa & Silva, 2018). Generally, the expression of IEGs in the SBN is higher in animals engaged in social interaction compared to isolated individuals (Delville et al., 2000; Cabrera-Álvarez et al., 2017; Loveland & Fernald, 2017). Notably, dominant individuals exhibit higher early gene expression within the SBN compared to non-interacting animals, with status-dependent activation patterns across the network (Kollack-Walker & Newman, 1995; Delville et al., 2000; Loveland & Fernald, 2017). However, it is important to note that the expression of IEGs, serving as nonspecific markers of activity, can also be enhanced in both dominants and subordinates immediately after an agonistic encounter, showing no discernible difference between them.

The neuropeptide arginine-vasotocin and its mammalian equivalent known as arginine-vasopressin (henceforth, AVT), play crucial roles in modulating social behaviour (Goodson & Bass, 2001; Albers, 2015). Moreover, these neuropeptide systems have been implicated in social status, as evidenced by distinct distribution patterns of AVT receptors in the social brain among dominant individuals (Cooper et al., 2005; Filby et al., 2010; Lema et al., 2015). However, the influence of AVT on social behaviour varies across species, sexes, physiological states, phenotypes, and social contexts (Insel & Young, 2000; Goodson et al., 2009; Godwin & Thompson, 2012; Caldwell, 2017; Johnson & Young, 2017). Additionally, it has been demonstrated in different vertebrate species that differential activation patterns of AVT neurons may happen between dominants and subordinates (Ferris et al., 1989; Larson et al., 2006; Greenwood et al., 2008; Hattori & Wilczynski, 2009;

Godwin & Thompson, 2012; Qiao et al., 2014; Lema et al., 2015; Teles et al., 2016; Terranova et al., 2016).

Numerous studies have provided evidence of the complex relationship between developmental and neuroendocrinological factors in shaping behaviours and social status across various taxa (Guhl, 1958; Gray, 1971; Liley & Stacey, 1983; Moore et al., 2005). It is now widely recognized that AVT plays a significant role in modulating social interactions, including aggression and territoriality (Dewan & Tricas, 2011; Kulczykowska & Kleszczyńska, 2014; Almeida & Oliveira, 2015; Teles et al., 2016; Loveland & Fernald, 2017). However, the neuropeptidergic control of AVT on the dominant-subordinate status has a complex nature. In fact, these hormones exert distinct actions depending on the social status, as evidenced by differential activation patterns of AVT neurons observed in dominants and subordinates across various vertebrate species (Ho et al., 2010; Kabelik et al., 2013; Terranova et al., 2016). Furthermore, although long-term status-dependent alterations are frequently observed in AVT cellular characteristics (Ferris et al., 1989; Larson et al., 2006), a clear understanding of the early patterns of neural activity among the nodes of the SBN and their effects on the development of social behaviour and dominance is still missing.

The daffodil cichlid (*Neolamprologus pulcher*) is a cooperatively breeding cichlid fish native to Lake Tanganyika in East Africa (Taborsky & Limberger, 1981; Balshine et al., 1998) that exhibits a complex social system based on dominance, involving frequent social interactions and specialized behaviours and signals (Wong & Balshine, 2011a). This species forms social groups comprising a dominant breeding pair and 1-20 non-breeding adult subordinates of both sexes, which assist the dominants in various tasks such as territory clearing, predator defence, and offspring care (Taborsky, 1984; Taborsky, 1985; Balshine et al., 2001; Heg et al., 2005; Desjardins et al., 2008). In daffodil cichlid groups, maintaining social rank within the dominance hierarchy involves a continuous process that relies on aggression, submission, and affiliative interactions (Wong & Balshine, 2011a). Vacancies in the dominance hierarchy can occur due to natural deaths or predation events, leading to competition among individuals, both from within and outside the group, for the newly available positions, particularly in high-ranking breeding positions (Balshine et al., 1998; Stiver et al., 2004; Fitzpatrick et al., 2008; Wong & Balshine, 2011b).

Previous studies have suggested that AVT may play a regulatory role in various behaviours of daffodil cichlids, including aggression, courtship, and parental care (Reddon et al., 2015; Reddon et al., 2017), indicating its potential importance in determining individuals' social status within the species. Notably, dominant daffodil cichlids were found to have higher expression levels of the AVT gene compared to subordinates (Aubin-Horth et al., 2007), although subordinates have higher concentrations of AVT in their brains compared to dominants (Reddon et al., 2015). While these status-dependent effects of AVT may imply contrasting activation patterns of the AVT system in dominants and subordinates, there has been a lack of assessment regarding short-term plastic changes in AVT neurons during agonistic interactions which may underpin the establishment of dominance hierarchies in daffodil cichlids.

A valuable approach to investigating the involvement of AVT neurons in the establishment of dominant-subordinate relationships is by examining the transient changes in AVT cell activity associated with the expression of IEGs. This immunohistochemical method has been employed in various vertebrate species (Goodson et al., 2009; Ho et al., 2010; Kabelik et al., 2013; Terranova et al., 2016), including teleost species (Pouso et al., 2023). In this study, we sought to investigate activation of AVT neurons in both dominant and subordinate individuals of the daffodil cichlids during agonistic encounters. To achieve this, we conducted controlled behavioural experiments involving an agonistic interaction for territorial dominance, which we predicted would elicit the activation of the AVT neurons, as determined by the co-expression of the IEG Phospho-S6 Ribosomal Protein (pS6) (Biever et al., 2015). By comparing brains with similar levels of social activation, we could be able to explore status-dependent, short-term plastic changes in AVT cells. In Chapter 4 of the present thesis, we demonstrated how AVT neuronal phenotypes in daffodil cichlids differ between statuses and are correlated with the levels of aggression. Our results showed that aggressive behaviours in dominant fish were positively correlated with the number of AVT positive parvo- and magnocellular neurons, but not in subordinate fish. Building on such evidence, we predict differences in the levels of AVT activated cells between dominant and subordinate individuals, with dominant individuals exhibiting higher number of AVT activated cells when compared to subordinate individuals. Specifically, we anticipate a higher number of AVT parvo- and magnocellular neurons activated in dominants fish with respect to the subordinate ones. Furthermore, we predicted a positive correlation between aggressive behaviours and AVT activated cells in dominant individuals.

5.3 MATERIALS AND METHODS

5.3.1 Study animals

The research subjects were laboratory reared daffodil cichlids (*Neolamprologus pulcher*) descended from fish originally captured on the southern shore of Lake Tanganyika, Africa. Fish were maintained in stock aquaria prior to the onset of the experiment, in mixed-sex groups with approximately 50 fish per aquarium (105 × 43 x 40cm, 180L). The aquaria were equipped with a heater, a thermometer, two powered filters, an air stone, and 3 cm of fine coral sand. Temperature was kept at 27 ± 1°C, with a 12:12 h light:dark day cycle and a 30min period of gradual transition from light to dark to simulate sunrise and sunset. The aquaria were regularly checked for water quality parameters and weekly cleanings were performed. Fish were fed daily with a mix of pellet cichlid food and dried flakes (Tetra Werke, Germany).

5.3.2 Experimental procedures

For our behavioural experiments based on territorial contests, we used 90L aquaria (53 × 43 x 38 cm) to form twelve groups. The aquaria were filled with only half of their intended capacity (~45L) and were supplied with approximately 3 cm of fine coral sand. Each aquarium was then subdivided into 3 equal partitions using opaque Plexiglass panels. Each of the lateral partitions was equipped with one foam filter, one heater, a thermometer, and a terracotta cave, which could be used by the fish as shelter. A terracotta cave was also positioned in the mid partition.

For each experimental group, one adult fish ("dominant") was haphazardly picked from one of the stock aquaria available and placed in one of the two lateral partitions. Then another adult fish was chosen from a different stock aquarium by matching the sex of the other fish introduced in the tank, so that the body size difference of around 15% could predict the contest outcome: the dominant fish (larger one) and the subordinate fish (smaller one) (Reddon et al., 2011). Sex was determined by examination of the genital papillae and then confirmed post-mortem. We selected 6 pairs of female fish and 6 pairs of male fish. The length of both the "dominant" and "subordinate" fish were recorded (mean ± SE standard length, measured from the tip of the snout to the end of the caudal peduncle: "dominant" = 4.99 ± 0.16 cm; "subordinate" = 4.2 ± 0.14 cm). The pairs were kept separated overnight, and the day after, before starting each trial, the shelters were carefully removed from the lateral partitions. After a minute, the opaque panels were removed so that the fish could interact and contest the shelter in the middle of the tank. Behavioural scoring was performed manually during the experiments by a trained observer (T.R.). We recorded all the aggressive behaviours (chases, rams, bites, head-down postures, frontal displays) performed by the dominant fish and received by the subordinate fish. For more details about these behaviours, see (Reddon et al., 2015).

After each experimental trial, the fish were immediately euthanised and their brain was processed for the immunohistochemistry with double-labelling for AVT and Phospho-S6 Ribosomal Protein Ser235/236 (pS6), a commonly used marker for neuronal activity (Biever et al., 2015). Briefly, we removed the fish from the aquaria and administered a lethal dose of tricaine methane sulfonate (MS222, 300mg/L, Sigma-Aldrich, Dorset, UK) dissolved in tank water and adjusted to pH 7.4. The fish were immersed in the MS222 for a maximum of 10 min and monitored until gill movement ceased. Afterwards, the brains of each fish were dissected and placed in a solution of 4% paraformaldehyde in 0.1M Phosphate buffered saline (1x PBS). The brains were stored overnight at 4°C. The next day, the brains were cryoprotected by immersing them in a 30% sucrose solution and keeping them overnight at 4°C. After cryoprotection, the brains were embedded in Optimal Cutting Temperature (OCT) compound in moulds and rapidly frozen using dry ice-chilled hexane. The whole brains were then sliced coronally into 30µm thick sections using a Leica CM3050s Cryostat and mounted on Superfrost Plus Gold slides (Fisher Scientific, Loughborough, UK) in two parallel series. The sections were stored at -20°C until they were ready to undergo immunohistochemistry processing.

5.3.3 Immunohistochemistry process

The sections were allowed to thaw and air-dry for 15 min, following which they were marked with a hydrophobic pen to prevent any liquid spills during the subsequent incubation steps. Afterwards, the slides were washed three times for five min each with 1x PBS. To block any nonspecific antibody binding, the slides were then placed in a moist chamber and incubated with 350ml of a blocking buffer consisting of 2% normal goat serum (Vector Laboratories, Peterborough, UK) diluted in 1x PBST (1x PBS + 0.1% Triton X-100) for 60 min. Following this step, the slides were incubated in an anti-pS6 (catalogue n. C.S.T. #2211, Cell Signaling Technology, Danvers, MA, USA) rabbit polyclonal antibody, optimised at a concentration of 1:100,000 in blocking buffer and stored at 4°C overnight in a moist chamber.

On the subsequent day, the slides underwent a triple wash in 1x PBST for 5 min, followed by an incubation in a biotinylated goat anti-rabbit IgG secondary antibody (catalogue n. BA-1000-1.5, Vector Laboratories, Inc., Newark, CA, USA) diluted in NS blocking buffer (concentration: 1:200) for 60 min at room temperature. After a triple wash in 1x PBST for 5 min, slides were incubated in an ABC-HRP solution (Vector Laboratories, Inc. Newark, CA, USA), composed of 1% avidin and 1% biotin HRP and diluted in blocking buffer, for 30 min. Slides were rinsed in 1x PBS 3 times for 5 min each, and then incubated in the dark in a biotinylated tyramide solution (1:50) diluted in tyramide signal amplification buffer (TSA Biotin Stand Alone Tyramide Kits, catalogue n. SAT700001KT, Perkin-Elmer, Waltham, MA, USA) for 30 min. Slides were rinsed again in 1x PBS 3 times for 5 min each and incubated in the dark with a streptavidin-fluorescent solution (DyLight 488 Streptavidin, catalogue n. SA-5488-1, Vector Laboratories, Peterborough, UK) diluted in 1x PBST (1:500) for 45 min. Slides were rinsed in 1x PBS another 5 times for 5 min each and, after this step, incubated in blocking buffer for 60 min. Finally, the slides were incubated in a rabbit α-Arginine Vasopressin (α-AVP, catalogue n. ABIN617884, Immunostar, Hudson, WI, USA) primary antibody (1:1000), diluted in blocking buffer and stored at 4°C overnight in a moist chamber. This AVT antibody was previously validated for use in daffodil cichlids in Chapter 4 of the present thesis.

On the final day of the process, the slides underwent a triple wash in 1x PBST for 5 min, followed by an incubation in the dark with Goat anti-Rabbit IgG H&L (Alexa Fluor™ 555, catalogue n. ab150078, Abcam, Cambridge, UK) secondary antibody (1:1000), diluted in blocking buffer for 1 hour in a moist chamber at room temperature. Subsequently, the slides were triple washed in 1x PBS and a single washed in 1x PBST, each time for 5 min, while still in the dark. Sections were then briefly dipped in de-ionized water to remove excess of salt, mounted with three/four drops of Fluoroshield + DAPI mounting medium (Abcam, Cambridge, UK), and sealed with clear nail varnish to prevent drying or moving of the sections under the microscope. The mounted slides were stored in a sealed container at 4°C to prevent photobleaching until the imaging process.

5.3.4 Imaging and measuring cells

The imaging process was performed with a Leica LMD6 fluorescent microscope using 20x magnification. Individual brain sections were imaged using GFP (absorption peak = 450-

490nm) for the pS6 positive cells, RHO (absorption peak = 541-551nm) for the AVT positive cells, and DAPI (absorption peak =340-380nm) fluorescence filters. Neurons were discriminated by manually identifying the cell type based on soma size and localization: parvocellular cells (Fig. 1A & Fig. 1B) were identified as densely-packed smaller neurons located in the anterior and ventral region of the preoptic area (POA) of the brain, magnocellular neurons (Fig. 1C & 1D) as mid-sized neurons located in the posterior and dorsal region throughout the POA, and gigantocellular neurons (Fig. 1E & 1F) as large neurons in the posterior and dorsal region of the POA. All the AVT cell types in the POA were counted independently and quantified manually during the imaging process.

A Leica LMD6 fluorescent microscope was used to perform the imaging procedures, using a 20x magnification. Individual brain sections were imaged using fluorescence filters GFP (absorption peak = 450-490nm) for the pS6 positive cells, RHO (absorption peak = 541-551nm) for the AVT positive cells, and DAPI (absorption peak =340-380nm) for overall fluorescence. The identification of neurons was carried out manually by discriminating cell types based on soma size and localization. The quantification of all AVT cell types within the POA was performed manually during the imaging process. Moreover, we independently counted all the single-labelled AVT cells and the double-labelled AVT+pS6 cells. Due to tissue loss during the sectioning process, one male dominant and the corresponding subordinate brain sample were not quantified.

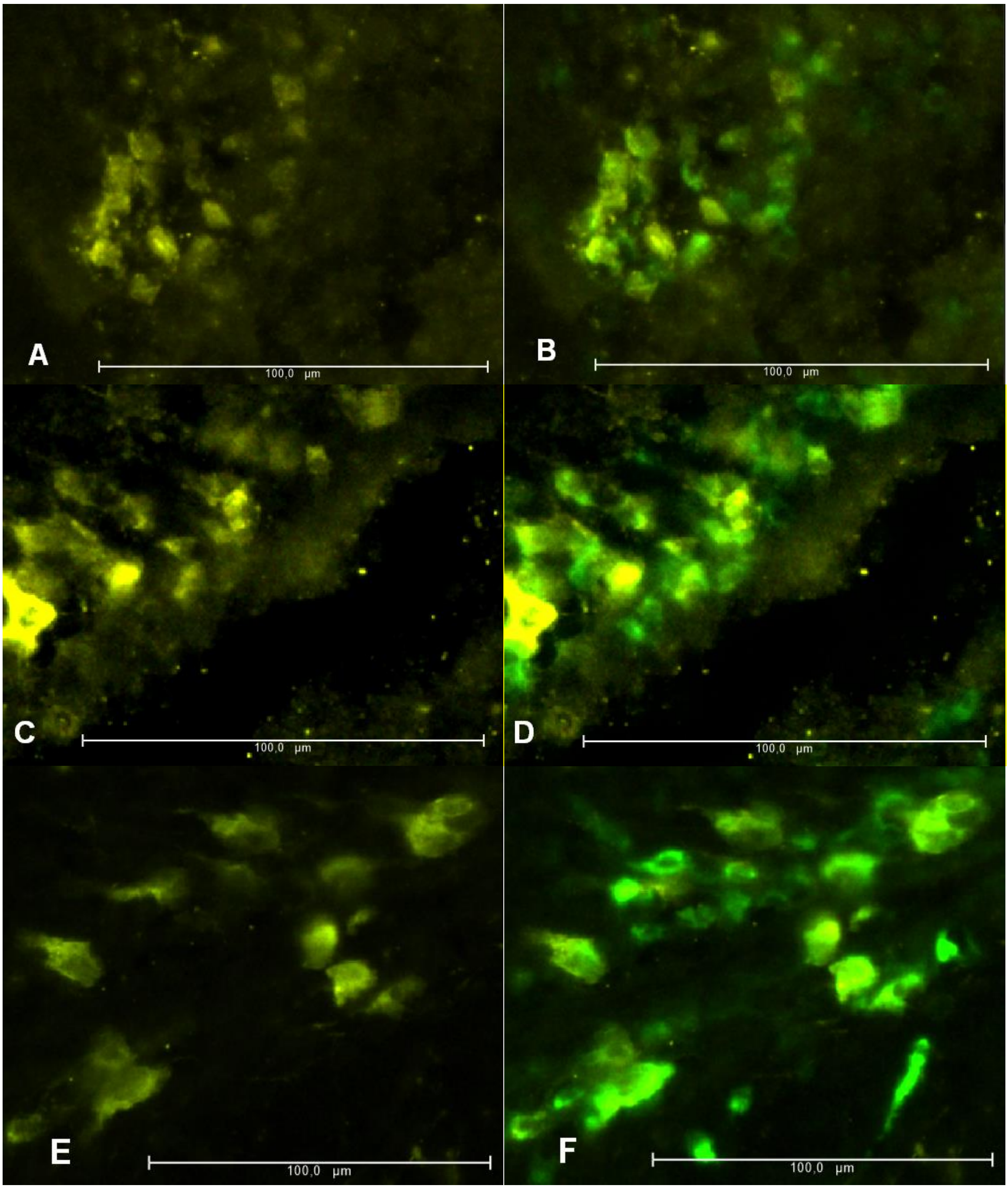

Figure 1: AVT parvocellular (A), AVT activated parvocellular (B), AVT magnocellular (C), AVT activated magnocellular (D), AVT gigantocellular (E), and AVT activated gigantocellular neurons (F) from a dominant male cichlid fish, imaged at 20x magnification. AVT neurons are depicted in yellow, and activated cells are depicted in green. The AVT activated neurons are noticeable by the overlay of the green fluorescent colour over the yellow basal colour.

5.3.5 Statistical analysis

To evaluate the potential differences in activated AVT cells between dominant and subordinate fish, we compared the cell measurements from the brain samples. First, to confirm the ranks of the focal fish, we compared the levels of aggression between dominant and subordinate fish using a paired samples *t*-test. Then, we conducted Linear Mixed Models (LMMs) to test differences between activated cells depending on the rank and sex of the focal fish. For the overall cell count and each cell type count, we divided the number of double-labelled cells by the number of single-labelled cells to get "AVT activated cell count indexes" for each count. We checked our data for any possible violations of statistical assumptions (that is, a normal distribution of residuals and homogeneity of variance across treatments) and proceeded to arcsine transformed the dataset before proceeding with the LMMs. We coded the activated cell count indexes as dependent variables and rank (that is, dominant or subordinate) and sex as predictors. In case of significant effects, a Sidak post hoc test for differences of means was used for pairwise comparisons. Then, to test whether the agonistic behaviours of our focal fish were correlated to the AVT activated cells, we conducted a Spearman's correlation test using the behavioural data that we gathered with the AVT activated cell count indexes. Specifically, for the dominant fish, we correlated the aggressive behaviours performed with all the AVT activated cell count indexes and, for the subordinate fish, we correlated the received aggressions with all the AVT activated cell count indexes. All statistics were performed using SPSS version 26.0 (IBM) for Windows.

5.3.6 Ethical note

Animal housing, handling, and study protocols were approved by the Liverpool John Moores Animal Welfare and Ethics Steering Group (approval number: AR_TR/2018-4) and adhered to the guidelines of the Animal Behaviour Society and the Association for the Study of Animal Behaviour. All fish were closely monitored for signs of injury. In case of fish showing serious signs of injuries and/or distress due to an overly aggressive contestant, the observer was instructed to immediately interrupt the ongoing experiments and separate the fish at once. Nonetheless, we confirm that no fish were injured or showed signs of distress during the status establishment procedure.

5.4 RESULTS

As expected, the dominant fish were more aggressive than the subordinate fish (t_{10}= 2.228, p = 0.005; aggressive behaviours ± SE: dominant: 22.81 ± 6.07; subordinate: 2.09 ± 0.88). The number of AVT activated cells were not affected by rank (overall cells: $F_{1, 5.82}$= 0.002, p = 0.962; parvocellular cells: $F_{1, 11}$= 0.147, p = 0.708; magnocellular cells: $F_{1, 5.79}$= 0.253, p = 0,633; gigantocellular cells: $F_{1, 5.90}$= 0.002, p = 0.964) or by sex (overall cells: $F_{1, 6.09}$= 2.111, p = 0.196; parvocellular cells: $F_{1, 11}$= 1.819, p = 0.205; magnocellular cells: $F_{1, 6.07}$= 2.246, p = 0.184; gigantocellular cells: $F_{1, 6.06}$= 1.530, p = 0.262).

We found that the correlations between aggressive behaviours and AVT activated cells in the dominant fish were never significant (overall cells: *r*(9) = 0.487, p = 0.128, Fig. 2A; parvocellular cells: *r*(9) = 0.524, p = 0.098, Fig. 2B; magnocellular cells: *r*(9) = 0.387, p = 0.239, Fig. 2C; gigantocellular cells: *r*(9) = 0.219, p = 0.517, Fig. 2D). For the subordinate fish, there was a non-significant positive correlation between AVT activated parvo-cellular cells and aggression received (*r*(9) = 0.072, p = 0.878), and non-significant negative correlations between all other AVT activated cell types and aggression received (overall cells: *r*(9) = -0.105, p = 0.759; magnocellular cells: *r*(9) = -0.273, p = 0.416; gigantocellular cells: *r*(9) = -0.075, p = 0.826).

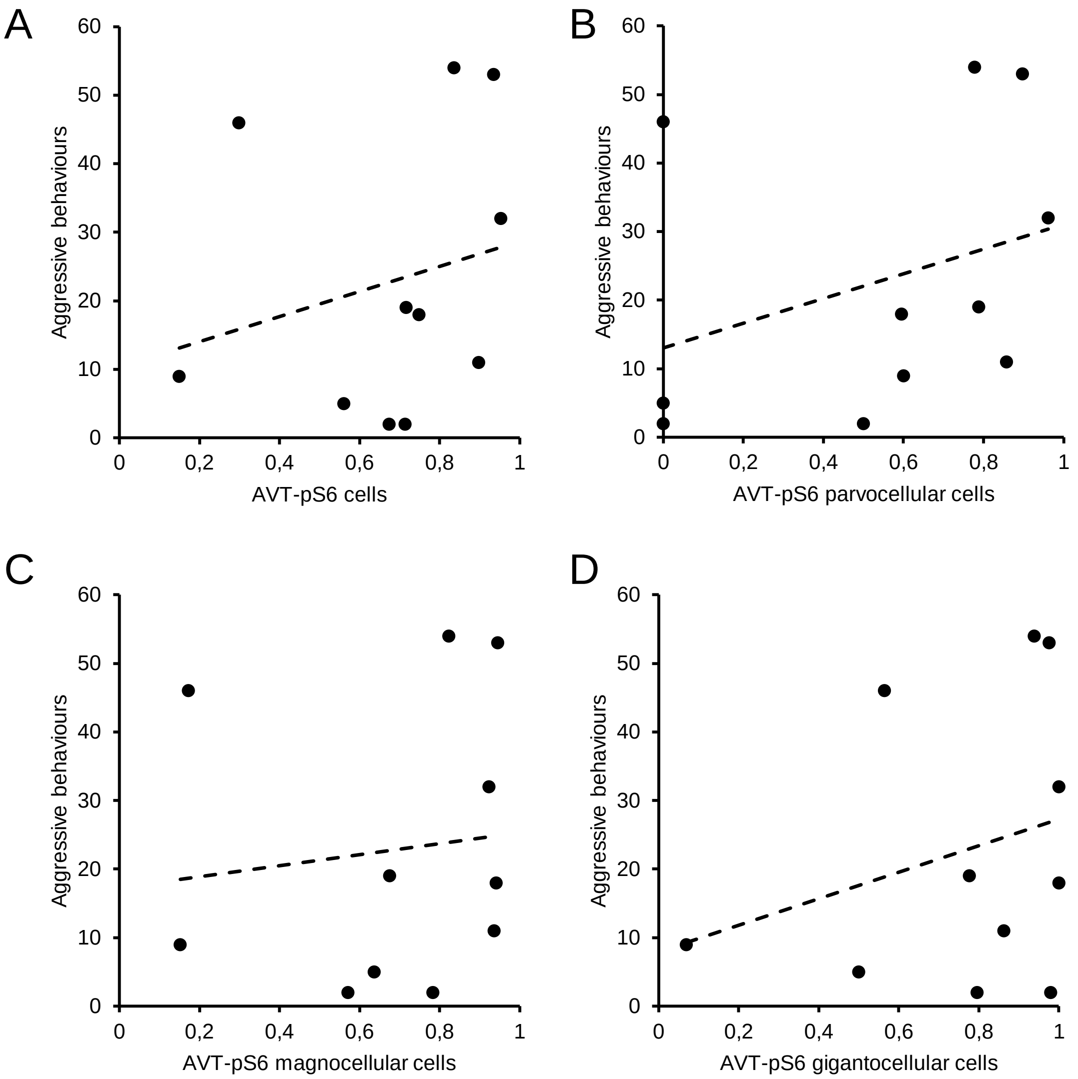

Figure 2. The aggressive behaviours of the dominant daffodil cichlids correlating with (A) the total AVT activated cells (AVT-pS6 cells), (B) the AVT activated parvocellular cells (AVT-pS6 cells), (C) the AVT activated magnocellular cells (AVT-pS6 cells), and (D) the AVT activated gigantocellular cells (AVT-pS6 cells). Linear best fit dotted lines are displayed..

5.5 DISCUSSION

In this study, we aimed to investigate the role of AVT in the formation of dominant-subordinate relationships in daffodil cichlids by combining a novel approach for the examination of daffodil cichlids neurophysiology and behaviour. Using a double-labeling TSA-based immunohistochemistry process, we were able to examine the immediate changes of AVT cell activity associated with the expression of the IEG pS6 right after territorial contest-based behavioral experiments. In contrast to previous studies highlighting long-term status dependent asymmetries of AVT neurons in teleosts (Larson et al., 2006; Greenwood et al., 2008; Iwata et al., 2010; Ramallo et al., 2012; Silva et al., 2013), and building on the findings reported in Chapter 4 of the present thesis, in which we demonstrated that AVT neuronal phenotypes in daffodil cichlids may differ between dominants and subordinates, we sought to identify early status-dependent variations in AVT cell activity directly following the establishment of dominance. After confirming that the status of the focal fish was correctly assigned by analysing the levels of aggression in our focal fish, we did not observe a difference in the number of AVT activated cells between dominant and subordinate individuals. Furthermore, our results showed that the correlation between aggressive behaviours and activation of AVT cells in dominant fish was not strong enough to be statistically significant.

Previous studies performed in different taxa, including teleost fish, have demonstrated that social stimuli elicit the activation of the SBN nodes, leading to an upregulation in the expression of early genes (Goodson et al., 2005; Fischer et al., 2018; Kabelik et al., 2018; Pouso et al., 2019). Notably, in animals engaged in fighting behavior, the expression of early genes is significantly higher compared to individuals not involved in a contest (Delville et al., 2000; Loveland & Fernald, 2017). However, it is worth noting that no discernible differences have been reported between dominants and subordinates (Kollack-Walker & Newman, 1995; Ramallo et al., 2012), likely due to similar activation of SBN in both contenders throughout the contest. Our findings seem to confirm this trend, as we could not identify any difference in AVT activated cells between dominant and subordinate daffodil cichlids. Indeed, we have to note that in our experiments we could not set a baseline level for the AVT activated cells by analysing brain tissue from individuals not involved in a contest. Daffodil cichlid are animals with a complex social system based on dominance hierarchies (Wong & Balshine, 2011a), therefore they rely on the presence of conspecifics. Isolated individuals would have not behaved in a natural way, and their stress levels may have had an impact on the expression of hormones in their brain and/or directly upon SBN activation. In fact, isolation may be considered a “social” treatment for a social fish, and therefore it may have potentially altered the activation of some nodes of the SBN (Christopher et al., 2021). Moreover, sampling individuals from the stock housing tanks may have not been optimal for setting the baseline levels for the AVT activated cells, as even in stock tanks we can observe frequent social interactions and some degree of dominance hierarchy (T.R. and A.R.R personal observations), although these interactions may differ by magnitudes from those showed in staged encounters.

The results from our experiments showed a non-significant pattern of positive correlations between aggressive behaviours and the number of AVT activated cells, regardless of the cell type, in dominant individuals immediately after the establishment of the dominance hierarchy. Moreover, we could not find a clear correlation between subordination and AVT in subordinate fish. It is worth noting that, in teleost fish, previous studies into nonapeptidergic cellular characteristics have primarily focused on long-term stable hierarchies (Larson et al., 2006; Greenwood et al., 2008; Iwata et al., 2010; Ramallo et al., 2012). Findings from such studies have revealed phenotype-dependent asymmetries in both the quantity and size of AVT neurons (Iwata et al., 2010; Ramallo et al., 2012), as well as variations in the levels of AVT positive cells, detected through immunohistochemistry (Ramallo et al., 2012) or in situ hybridization (Greenwood et al., 2008). Nonetheless, the findings seem to be species and/or context-dependent: in some cases, there seems to be a positive correlation between AVT cells and dominance (Larson et al., 2006; Iwata et al., 2010), in other cases, results showed a negative correlation between the two factors (Pouso et al., 2023), and some other times, such correlation was not found (Ramallo et al., 2012).

We must acknowledge that, in this study, fewer AVT parvocellular cells were found from the immunohistochemistry performed than the expected numbers, based on the results from both Chapter 4 analyses and a previous study on daffodil cichlids (Reddon et al., 2017). This may suggest that there could be some kind of signal interference during the immunohistochemistry process, possibly due to the double-labelling TSA-based approach, that may have interfered with AVT staining, at least for the smallest and most densely packed parvocellular cells. As we cannot rule out this as a potential issue in biasing our results, we suggest that, for future experiments, other methods for visualising AVT cells rather than double labeling immunohistochemistry should be employed. For example, *in situ* hybridisation with RNAscope (Wang et al., 2012) in conjunction with immunohistochemisty may be an alternative to double labeling with immunohistochemistry alone. Alternatively, it would be worth testing other AVT and pS6 antibodies from different hosts, which in turn would allow us to avoid the use of the TSA-based approach.

In conclusion, we focused on examining the transient changes in AVT cell activity associated with the expression of early genes after a territorial contest-based behavioural test. We expected that differences in the AVT cell activity between individuals of different ranks would initiate the processes that lead to long term changes in neuronal phenotypes, thereby complementing the findings from Chapter 4. This is, to our knowledge, the first study in daffodil cichlids which involves the use of double-labeling TSA-based immunohistochemistry processes and IEGs for investigating the role of AVT in the establishment of dominant-subordinate relationships. Our results show a pattern of positive correlations, albeit not significant, between aggressive behaviours and AVT activated cells in dominant daffodil cichlids. Overall, our experimental design will be helpful to inform further experiments involving such an integrative and multidisciplinary approach which encompasses immunohistochemistry methods and behavioural studies.

Chapter 6: DISCUSSION

6.1 GENERAL OVERVIEW

The objective of this research was to investigate the mechanisms underlying the expression of conflict management and agonistic behaviours in the daffodil cichlid (*Neolamprologus pulcher*), and we focused on the role of submissive signalling, the effect of the environment, and the involvement of the hormone arginine vasotocin (AVT) in regulating social behaviours and the development of dominance hierarchies.

Conflict management behaviours usually encompasses a complex interplay between dominance hierarchies, kinship, and other behavioural adaptations, such as aggression, submission, and cooperation (Aureli & de Waal, 2000), and examples among vertebrates are found across different species of mammals (Macdonald, 1983; Flack & De Waal, 2004), birds (Clayton & Emery, 2007; Silk, 2007) and fishes (Bshary et al., 2002). Understanding how social animals may handle conflicts is therefore an essential milestone in developing a thorough comprehension of social group dynamics, and to accomplish this, it is crucial to identify the fundamental biological factors that might influence the manifestation of conflict management behaviours. The daffodil cichlid was the ideal animal model for integrative social behaviour research, as it offered a unique opportunity to perform highly controlled behavioural studies in a vertebrate species with a complex dominance-based social hierarchy (Taves et al., 2009; Le Vin et al., 2010; Wong & Balshine, 2011a; Bruintjes & Radford, 2013; Dey et al., 2015; Reddon et al., 2015).

First, we focused on analysing the role of a putative submissive signal performed by daffodil cichlids, the head-up display (HUD) (Chapter 2), and the results from the experiments demonstrated that when a subordinate individual produced a HUD in response to aggression from a dominant male individual, the dominant reduced the frequency of aggression towards the signalling subordinate. HUDs were also rarely produced by the subordinate without any aggression, and the number of HUDs strongly correlated with the amount of aggressive behaviours received by the subordinate.

In chapter 3, we investigated how ecological contexts may affect the expression of agonistic behaviours in daffodil cichlids by manipulating the environment in which they lived in and altering the number of available shelters. We demonstrated that the aggression received by the subordinate from the dominant depended on the experimental condition, with fish receiving more aggression in the most enriched condition. Submissive behaviours and the fleeing of the subordinate fish varied depending on the aggression received and they were influenced by the experimental condition. Finally, the two subordinate fish responded differently to the aggressions received, with the larger subordinate submitting more and fleeing less than the smaller subordinate.

Chapter 4 evaluated the status-dependent differences in AVT neuronal phenotypes and their effect on behavioural responses in daffodil cichlid. We aimed to explore AVT neuronal phenotype differences between ranks and sexes by evaluating the correlations between behaviours and the expression of AVT neurons in the pre-optic area of the brain. We showed that dominants had larger AVT cells overall compared to subordinates, but subordinates had more parvocellular neurons than dominants. Aggression in dominants was positively correlated with the parvo- and magnocellular cell counts, however, except for positive correlation between aggression levels and the parvocellular neurons in the females, no correlations were found between aggressive behaviours and cell areas in dominants nor between submissive behaviours and AVT neurons in subordinates.

Finally, building on the findings of Chapter 5, we explored the role of AVT in the establishment of dominance hierarchies in daffodil cichlids. Specifically, in the last experimental chapter, we performed a behavioural test based on a status contest, in which two sex-paired but sized-mismatched daffodil cichlids were placed in the same experimental tank and, after the dominant-subordinate relationship was established, we examined the transient variations in AVT cell activity associated with the expression of the IEG pS6. Although we did not find any difference between AVT activated cells in dominant and subordinate fish, the correlations between aggressive behaviours and AVT activated cells showed a trend of moderately positive correlations, albeit not significant possibly due to a low sample size.

6.2 SUBMISSIVE SIGNALS IN DAFFODIL CICHLIDS

Conflicts among members of the same species are an inevitable occurrence for most animals, as they need to compete for limited resources essential for their survival and reproductive success However, conflicts are costly, both in terms of time, energy, and potential injuries (Huntingford et al., 1987; Hardy & Briffa, 2013), and often these costs are not that different between the winner and the loser of a contest (Geist, 1974; Enquist & Leimar, 1990; Brick, 1998; Maan et al., 2001; Morrell et al., 2005; Earley et al., 2006; Copeland et al., 2011). Submission signals are a particularly salient example of an adaptation for conflict resolution (Kutsukake & Clutton-Brock, 2006; Kutsukake & Clutton-Brock, 2008) used to pre-emptively avoid or end a contest while reducing the chance of receiving further aggressions (Reddon et al., 2022). With respect to the daffodil cichlids, it has been demonstrated that the tactical use of submission signals as is a key element of social competence (Bergmüller et al., 2005; Taborsky et al., 2012; Fischer et al., 2017). Within the ethogram of the daffodil cichlids, the head up displays have long been assumed to have a submission signalling function, but this study is the first demonstration that the head up display has this effect on the receiver of this behaviour.

In our experiments, it was also noted that the HUDs themselves also exhibited some levels of variation, and it was also accompanied by tail/body quivering, a submissive behaviour present in the ethogram of the species (Reddon et al., 2015). For instance, the extent to which some fish raised their head in the water column was ranging from a slight upward pivot to the fish adopting a nearly perpendicular position. Indeed, this variation appears to modulate the amplitude of the submissive signal, as proposed in a recently published study which investigated this aspect of the daffodil cichlid communication (see Appendix 1). In particular, Manara et al. (Manara et al., 2023) analysed the behaviour of subordinate daffodil cichlids in response to the aggression of dominant individuals. When faced with overt aggression, fleeing was the predominant response of the subordinate, whereas submission signals were more frequently observed in response to restrained aggression. The frequency of head up displays in subordinates was influenced by the number of aggressive acts received from the dominants, while the angle of head up display varied depending on the type of aggressive behaviour received. Both HUD and the tail-quivering displays were employed in response to both restrained and overt aggression. Furthermore, the head up displays, but not the tail-quivering, significantly correlated with the aggression received. The act of tail-quivering could have been repurposed from other communication contexts to function as a submissive-affiliative gesture, as in fact it acts as a signal during courtship among various cichlid species (Baerends & Baerends-van Roon, 1950; Barlow, 2008). In contrast, the head up display seems to be more specific to the daffodil cichlid. These findings indicate that the head up displays may actually serve as a more specific and nuanced signal of submission, whereas the tail-quivering displays may serve other social functions. Together, these two studies help elucidating the degrees of communication in daffodil cichlids and contribute to our improved understanding of the utilization of graduated submission signals in this highly social species.

6.3 ENVIRONMENT AND ITS EFFECTS ON AGONISTIC BEHAVIOURS

To gain a better insight into how conflict is handled within intricate social groups, it is crucial to grasp the factors that impact the use of agonistic behaviours within the group. Among these factors, the structure of the physical environment plays a significant ecological role as it can influence the competition for resources, ultimately determining the success or failure of a social group (Bell et al., 2012). In a previous study, behavioural data collected in laboratory reared groups of daffodil cichlids was used to evaluate which factors would influence the expression of submissive behaviours (Reddon et al., 2019). Building upon the findings from this study, in chapter 3, we further explored the impact of manipulating the physical environment of daffodil cichlids on the expression of agonistic behaviours. Our experiments revealed that the aggression directed towards helpers by breeders varied based on the experimental conditions, with fish experiencing more aggression in the enriched environment. Both submissive behaviours and the fleeing response of the subordinate fish were influenced by the aggression received, and were affected by the shelter treatment, confirming the results from Reddon et al. (2019).

In daffodil cichlids, subordinate individuals have several strategies to avoid aggression from dominant individuals: for example, they can perform cooperative behaviours, escape directly from aggression, or display submission signals, such as head-up submissive postures or tail quivering, towards aggressing groupmates (Taborsky, 1985; Wong & Balshine, 2011a). Retreating from a conflict and moving to a new location might seem like the easiest escape strategy for these animals. However, in daffodil cichlids, this option is not always feasible due to factors, such as group membership and social identity, that play significant roles in providing protection against high predation risk (Wong, 2010; Hick et al., 2014; Balshine et al., 2017). Additionally, other ecological limitations on movement, such as a saturated habitat or the absence of a safe location within reach, may further hinder their ability to flee (Wong, 2010; Batista et al., 2012). Considering these constraints, submission signals must play a crucial role in the social interactions of daffodil cichlids, particularly when fleeing options are limited (Wong, 2010).

Notably, although both subordinate fish in our experiments received similar levels of aggression, they responded differently to these aggressions, with the larger subordinate that tended to submit more frequently but to flee less when compared to the smaller subordinate. In various animal species, body size plays a critical role in determining fighting ability, and smaller individuals usually find it difficult to come out victorious in such confrontations (Parker, 1974). Experiments performed in daffodil cichlids indicates that, in dyadic contests, the fish with a body size difference of 5% or more is usually the winner, implying that smaller fish have a low likelihood of winning such conflicts, and also the individual’s perception of such chance may be minimal (Reddon et al., 2011). In contrast to the results from the study that prompted our experiments (Reddon et al., 2019), submissive behaviours were not often used by the smaller fish in response to aggression. Instead, our findings are more consistent with other studies, in which subordinates tend to display higher levels of submission when they are similar in size to the dominant breeder (Hamilton et al., 2005), since closely matched individuals should be more likely to interact with each other (Dey et al., 2013). Furthermore, smaller and weaker fish may face an increased risk of injury when confronted by larger and stronger individuals (Lane & Briffa, 2017), which raises the potential costs of such interactions. Hence, the smaller helpers might have found it more advantageous to flee from aggression rather than remaining and submitting, while also taking the risk of being attacked again.

Indeed, the manipulation of the physical environment did not affect the workload, leading to the conclusion that helpers in our experiments might have favoured an avoidance strategy rather than resorting to workload or submissive behaviours to reduce aggression. While one could argue that idling or neglecting helping duties might be seen as the opposite of the workload measure, in our experiments we were unable to accurately measure idling behaviours, such as standing still in floating shelters or behind tank furniture to avoid aggression from the breeders. In future experiments, it would be worth to evaluate such idling behaviours when observing the focal groups. To better test the helping tendencies and the workload behaviours of subordinate daffodil cichlids, we could further manipulate the environment by adding extra coral sand to the tanks in which the focal groups are housed, potentially filling up the nesting shelters, or placing an egg predator in a transparent tube nearby the nests. We could then perform behavioural observations of all the helping behaviours performed by the subordinate individuals following such treatments, similar to previous studies performed in the wild (Bruintjes & Taborsky, 2011; Reyes-Contreras et al., 2023).

6.4 NONAPEPTIDES AND AGONISTIC BEHAVIOURS

Nonapeptides are a class of neuropeptides found in vertebrates that exhibits high levels of conservation among different taxa, and they play crucial roles in various physiological processes such as cardiovascular function, osmoregulation, and stress (Banerjee et al., 2017). Furthermore, nonapeptides are involved in the regulation of social behaviours in animals (Bass & Groberb, 2001; Goodson & Bass, 2001; Goodson et al., 2003; Balment et al., 2006; Thompson et al., 2006; Godwin & Thompson, 2012), and they may also promote offensive aggressive behaviours (Ferris & Delville, 1994), social avoidance (Thompson & Walton, 2004), and aggressive responses to perceived threat (De Dreu et al., 2010). Among these nonapeptides, arginine vasotocin (AVT), the homologue of mammalian arginine vasopressin, serves as both a neurotransmitter and a neuromodulator in the central nervous system of teleost fish (Kulczykowska, 2008; Goodson & Thompson, 2010; Godwin & Thompson, 2012). Variations in social behaviours have been linked to the differential distribution, both within and between species, of AVT neural elements in several vertebrates, therefore gaining a thorough understanding of nonapeptide-producing cells in the brain could provide insights into the influence of AVT on social behaviour and shed light on hypotheses related to brain evolution and function (Godwin & Thompson, 2012; Thompson & Walton, 2013).

In chapter 4 we sought to use the daffodil cichlids as a model species for gaining a better understanding of the role of AVT in the expression of agonistic behaviours. Our study revealed that dominant individuals had larger AVT cells in general, and dominants also possessed a higher number of parvocellular neurons compared to subordinates. We also observed a positive correlation between aggression levels in dominants and the counts of parvo- and magnocellular cells. Our findings are in contrast with the results from a previous study in which subordinate daffodil cichlids exhibited higher levels of bioactive AVT in their brains compared to the dominant individuals (Reddon et al., 2015), even though in daffodil cichlids dominant individuals seem to have higher brain AVT gene expression compared to subordinates (Aubin-Horth et al., 2007).

It should be noted that the levels of gene expression might not consistently align with the ultimate concentrations of the bioactive nonapeptide. This discrepancy can be attributed to the multiple steps in the process between mRNA production and the final generation of the bioactive nonapeptide. Furthermore, differences in AVT production usually correlate with variations in gene expression, while variances in peptide availability may indicate variations in storage levels (Almeida et al., 2012; Reddon et al., 2015). Furthermore, in Reddon et al. (2015), the authors conducted a direct measurement of free biologically available nonapeptides in the brains, while most of the research on nonapeptide levels in fish brains, including our experiments, has relied on neuroanatomical or genetic indicators to assess the levels of nonapeptides (Aubin-Horth et al., 2007; Greenwood et al., 2008; Filby et al., 2010). The contrasting patterns observed in studies that utilized different methods highlight the importance of examining biologically available free nonapeptides alongside genomic and neuroanatomical techniques. These discrepancies also imply that incorporating multiple methodologies is essential in future investigations on the role of nonapeptides in the expression of behaviours.

With respect to the analyses of subordinate brain samples, we did not find any significant correlations between submissive behaviours and AVT neurons. Despite the lack of correlation between AVT and submissive behaviours in our study, it is known that isotocin (IT), the teleost homologue of the nonapeptide oxytocin, plays a significant role in inducing submissive behaviours within daffodil cichlids (Reddon et al., 2012; Hellmann et al., 2015). As our results from the experiments in Chapter 3 indicate a variation in submissive behaviours depending on the rank of the subordinate individuals, in future studies it would be worth evaluating the different neuronal circuitries involving IT and the expression of submissive behaviours, and possibly assess any IT neuronal phenotypes in subordinate daffodil cichlids. Previous studies demonstrated the existence of helpful behavioural types in daffodil cichlids (Heg et al., 2011; Le Vin et al., 2011; Riebli et al., 2011), and it was also demonstrated in a laboratory environment experiment that subordinate daffodil cichlids may either specialize to be submissive helpers or a less-submissive fish with a more marked tendency to disperse (Fischer et al., 2017). Within this framework, it would be interesting to evaluate the possible involvement of nonapeptides (AVT and IT) in the expression of these putative alternative behavioural types.

6.5 TERRITORIAL DOMINANCE AND VASOTOCIN

Agonistic behaviours are crucial in determining social hierarchies among animals. The capacity to recognize both one's own social standing and that of others is vital for group-living creatures to avoid persistent and resource-intensive conflicts (Maynard Smith & Parker, 1976). However, the behavioural and neurological mechanisms responsible for establishing dominance hierarchies in social animals remain poorly understood. In chapter 4, we observed differences in AVT cell counts between dominant and subordinate individuals, and we also noted that aggressive behaviours in dominants showed a positive correlation with both AVT parvocellular and magnocellular cell counts. Building on this evidence, we sought to understand whether AVT was involved in the establishment of dominance hierarchies, and to this aim, we investigated the AVT cell activity associated with the expression of immediate early genes (IEGs) associated with neuronal activity after a learning process or a behavioural-state response (Long & Salbaum, 1998; Thomas & Brooks-Kayal, 2013; Barbosa & Silva, 2018). Unfortunately, the results of our experiments were inconclusive, possibly due to the methodology we followed or as a result of insufficient power. In particular, in our experiments we employed a double-labelling methodology based on tyramide signal amplification (TSA), which required that additional passages would be added to our standard AVT immunohistochemistry processes. Indeed, we cannot exclude that the double-labelling through the TSA process interfered with the staining of AVT cells, as we did find fewer AVT parvocellular cells in the processed samples from these experiments than the ones from Chapter 4. However, TSA protocols have been employed along with immunohistochemistry processes in other fish species, such as the zebrafish (*Danio rerio*), to characterise the co-localization of tyrosine hydroxylase and the IEG *egr1* (Kress & Wullimann, 2012). Noteworthy, in this study on zebrafish, the authors employed *in situ* hybridisation, which may be a suggestive alternative to immunohistochemistry for future investigations involving IEGs in daffodil cichlids.

Another possible bias to our experimental setup was the lack of a basal level for the AVT activated cells. In a recent study on the weakly electric fish *Gymnotus omarorum*, the authors performed a similar experiment based on a territorial contest to understand the role of nonapeptide hormones during the establishment of dominance (Pouso et al., 2023). In their experiments, they established a baseline level of activated neurons by performing a control test, in which the focal fish were not allowed to interact and therefore have a contest for a territory. Although highly territorial, *G. omarorum* individuals do not live in social groups (Richer-de-Forges et al., 2009), nor they rely on other groups member like the daffodil cichlids do (Wong, 2010; Hick et al., 2014; Balshine et al., 2017). Daffodil cichlid individuals kept in isolation may not exhibit natural behaviours, and their stress levels could potentially influence the expression of hormones in their brain and directly affect the activation of the SBN, therefore we opted for an experiment without a non-social condition. Nonetheless, it would be useful in the future, to conceptualise an experiment for understanding the baseline levels of AVT activated neurons in status free individuals.

6.6 FINAL THOUGHTS

In this thesis, we employed various perspectives to delve into the intricacies of the daffodil cichlids’ agonistic behaviours and the regulatory mechanisms associated with them. We first elucidated the aspects of submissive signalling in subordinate daffodil cichlids. We discovered that the environment plays a significant role in modulating aggressive behaviour of the dominant individuals, as well as the nonapeptide hormone vasotocin. However, neither the correlation between submissive behaviours and nonapeptides, nor the precise mechanisms through which the brain coordinates the response to the establishment of dominance hierarchies, have been fully uncovered. These questions, along with other findings from this thesis, highlight the need for further exploration of the nature of agonistic behaviours in daffodil and merit additional investigations towards the aim of a more comprehensive understanding of conflict management in social species.

REFERENCES

Albers, H.E. (2012). The regulation of social recognition, social communication and aggression: vasopressin in the social behavior neural network. — Horm. Behav. 61, 283-292.

Albers, H.E. (2015). Species, sex and individual differences in the vasotocin/vasopressin system: Relationship to neurochemical signaling in the social behavior neural network. — Front. Neuroendocrinol. 36, 49-71.

Alexander, R.D. (1974). The evolution of social behavior. — Annu. Rev. Ecol. Syst. 5, 325-383.

Almeida, O., Gozdowska, M., Kulczykowska, E. & Oliveira, R.F. (2012). Brain levels of arginine–vasotocin and isotocin in dominant and subordinate males of a cichlid fish. — Horm. Behav. 61, 212-217.

Almeida, O. & Oliveira, R.F. (2015). Social status and arginine vasotocin neuronal phenotypes in a cichlid fish. — Brain Behav. Evol. 85, 203-213.

Angiulli, E., Pagliara, V., Cioni, C., Frabetti, F., Pizzetti, F., Alleva, E. & Toni, M. (2020). Increase in environmental temperature affects exploratory behaviour, anxiety and social preference in *Danio rerio*. — Sci. Rep. 10, 5385.

Anholt, B.R. (1990). An Experimental Separation of Interference and Exploitative Competition in Larval Damselfly. — Ecology 71, 1483-1493.

Arechavala-Lopez, P., Diaz-Gil, C., Saraiva, J.L., Moranta, D., Castanheira, M.F., Nuñez-Velázquez, S., Ledesma-Corvi, S., Mora-Ruiz, M.R. & Grau, A. (2019). Effects of structural environmental enrichment on welfare of juvenile seabream (*Sparus aurata*). — Aquac. Rep. 15, 100224.

Arnold, C. & Taborsky, B. (2010). Social experience in early ontogeny has lasting effects on social skills in cooperatively breeding cichlids. — Anim. Behav. 79, 621-630.

Arnold, K.E. & Owens, I.P. (1999). Cooperative breeding in birds: the role of ecology. — Behav. Ecol. 10, 465-471.

Aubin-Horth, N., Desjardins, J.K., Martei, Y.M., Balshine, S. & Hofmann, H.A. (2007). Masculinized dominant females in a cooperatively breeding species. — Mol. Ecol. 16, 1349-1358.

Aureli, F., Cords, M. & Van Schaik, C.P. (2002). Conflict resolution following aggression in gregarious animals: a predictive framework. — Anim. Behav. 64, 325-343.

Aureli, F. & De Waal, F.B.M. (2000). Natural conflict resolution. — University of California Press, Berkeley, CA, USA.

Backström, T. & Winberg, S. (2017). Serotonin coordinates responses to social stress—what we can learn from fish. — Front. Neurosci. 11.

Baerends, G.P. & Baerends-Van Roon, J. (1950). An introduction to the study of the ethology of the cichlid fishes. — Brill, Leiden, Netherlands.

Balment, R.J., Lu, W., Weybourne, E. & Warne, J.M. (2006). Arginine vasotocin a key hormone in fish physiology and behaviour: A review with insights from mammalian models. — Gen. Comp. Endocrinol. 147, 9-16.

Balshine, S., Leach, B., Neat, F., Reid, H., Taborsky, M. & Werner, N. (2001). Correlates of group size in a cooperatively breeding cichlid fish (*Neolamprologus pulcher*). — Behav. Ecol. Sociobiol. 50, 134-140.

Balshine, S., Neat, F.C., Reid, H. & Taborsky, M. (1998). Paying to stay or paying to breed? Field evidence for direct benefits of helping behavior in a cooperatively breeding fish. — Behav. Ecol. 9, 432-438.

Balshine, S., Wong, M.Y.L. & Reddon, A.R. (2017). Social motivation and conflict resolution tactics as potential building blocks of sociality in cichlid fishes. — Behav. Processes 141, 152-160.

Balzarini, V., Taborsky, M., Villa, F. & Frommen, J.G. (2017). Computer animations of color markings reveal the function of visual threat signals in *Neolamprologus pulcher*. — Curr. Zool. 63, 45-54.

Banerjee, P., Joy, K.P. & Chaube, R. (2017). Structural and functional diversity of nonapeptide hormones from an evolutionary perspective: A review. — Gen. Comp. Endocrinol. 241, 4-23.

Barbosa, F.F. & Silva, R.H. (2018). Immediate-early gene expression in neural circuits related to object recognition memory. — In: Handbook of Behavioral Neuroscience, Elsevier, London, UK, p. 261-271.

Barley, A.J. & Coleman, R.M. (2010). Habitat structure directly affects aggression in convict cichlids *Archocentrus nigrofasciatus*. — Curr. Zool. 56, 52-56.

Barlow, G. (2008). The cichlid fishes: nature's grand experiment in evolution. — Basic Books, New York City, NY, USA.

Barreto, R.E., Carvalho, G.G.A. & Volpato, G.L. (2011). The aggressive behavior of Nile tilapia introduced into novel environments with variation in enrichment. — Zoology 114, 53-57.

Bartolomucci, A., Palanza, P., Gaspani, L., Limiroli, E., Panerai, A.E., Ceresini, G., Poli, M.D. & Parmigiani, S. (2001). Social status in mice: behavioral, endocrine and immune changes are context dependent. — Physiol. Behav. 73, 401-410.

Bass, A.H. & Groberb, M.S. (2001). Social and neural mofdulation of sexual plasticity in teleost Fish. — Brain Behav. Evol. 57, 293-300.

Bates, D., Mächler, M., Bolker, B. & Walker, S. (2015). Fitting linear mixed-effects models using lme4. — J. Stat. Softw. 67, 1 - 48.

Batista, G., Zubizarreta, L., Perrone, R. & Silva, A. (2012). Non-sex-biased Dominance in a Sexually Monomorphic Electric Fish: Fight Structure and Submissive Electric Signalling. — Ethology 118, 398-410.

Bauer, E.B. & Smuts, B.B. (2007). Cooperation and competition during dyadic play in domestic dogs, *Canis familiaris*. — Anim. Behav. 73, 489-499.

Beaulieu, M., Mboumba, S., Willaume, E., Kappeler, P.M. & Charpentier, M.J.E. (2014). The oxidative cost of unstable social dominance. — J. Exp. Biol. 217, 2629-2632.

Beeching, S.C. (1995). Colour pattern and inhibition of aggression in the cichlid fish *Astronotus ocellatus*. — J. Fish Biol. 47, 50-58.

Bell, S.S., Mccoy, E.D. & Mushinsky, H.R. (2012). Habitat structure: the physical arrangement of objects in space. — Springer Science & Business Media.

Bergmüller, R., Heg, D. & Taborsky, M. (2005). Helpers in a cooperatively breeding cichlid stay and pay or disperse and breed, depending on ecological constraints. — Proc. R. Soc. Lond., Ser. B: Biol. Sci. 272, 325-331.

Bergmüller, R. & Taborsky, M. (2005). Experimental manipulation of helping in a cooperative breeder: helpers ‘pay to stay’by pre-emptive appeasement. — Anim. Behav. 69, 19-28.

Bernstein, I.S. (1981). Dominance: The baby and the bathwater. — Behav. Brain Sci. 4, 419-429.

Bhat, A., Greulich, M.M. & Martins, E.P. (2015). Behavioral Plasticity in Response to Environmental Manipulation among Zebrafish (*Danio rerio*) Populations. — PLoS ONE 10, e0125097.

Biever, A., Valjent, E. & Puighermanal, E. (2015). Ribosomal Protein S6 Phosphorylation in the Nervous System: From Regulation to Function. — Front. Mol. Neurosci. 8.

Bilhete, C. & Grant, J.W.A. (2016). Short-term costs and benefits of habitat complexity for a territorial Fish. — Ethology 122, 151-157.

Biro, P.A. & Stamps, J.A. (2010). Do consistent individual differences in metabolic rate promote consistent individual differences in behavior? — Trends Ecol. Evol. 25, 653-659.

Boinski, S. & Garber, P.A. (2000). On the move: how and why animals travel in groups. — University of Chicago Press, Chicago, IL, USA.

Bradbury, J. (1981). The evolution of leks. — In: Natural selection and social behavior. Blackwell Scientific, Oxford, UK, p. 138–169.

Brick, O. (1998). Fighting behaviour, vigilance and predation risk in the cichlid fish *Nannacara anomala*. — Anim. Behav. 56, 309-317.

Brown, J.H. (1971). Mechanisms of competitive exclusion between two species of chipmunks. — Ecology 52, 305-311.

Brown, J.L. (2014). Helping communal breeding in birds: Ecology and evolution. — Princeton University Press, Princeton, NJ, USA.

Bruintjes, R. & Radford, A.N. (2013). Context-dependent impacts of anthropogenic noise on individual and social behaviour in a cooperatively breeding fish. — Anim. Behav. 85, 1343-1349.

Bruintjes, R. & Taborsky, M. (2011). Size-dependent task specialization in a cooperative cichlid in response to experimental variation of demand. — Anim. Behav. 81, 387-394.

Bshary, R. & Oliveira, R.F. (2015). Cooperation in animals: toward a game theory within the framework of social competence. — Curr. Opin. Behav. Sci. 3, 31-37.

Bshary, R., Wickler, W. & Fricke, H. (2002). Fish cognition: a primate's eye view. — Anim. Cogn. 5, 1-13.

Burgdorf, J., Kroes, R.A., Moskal, J.R., Pfaus, J.G., Brudzynski, S.M. & Panksepp, J. (2008). Ultrasonic vocalizations of rats (*Rattus norvegicus*) during mating, play, and aggression: Behavioral concomitants, relationship to reward, and self-administration of playback. — J. Comp. Psychol. 122, 357.

Buston, P.M. (2003). Forcible eviction and prevention of recruitment in the clown anemonefish. — Behav. Ecol. 14, 576-582.

Cabrera-Álvarez, M.J., Swaney, W.T. & Reader, S.M. (2017). Forebrain activation during social exposure in wild-type guppies. — Physiol. Behav. 182, 107-113.

Caldwell, H.K. (2017). Oxytocin and vasopressin: powerful regulators of social behavior. — Neuroscientist 23, 517-528.

Caldwell, H.K., Lee, H.-J., Macbeth, A.H. & Young Iii, W.S. (2008). Vasopressin: behavioral roles of an “original” neuropeptide. — Prog. Neurobiol. 84, 1-24.

Cant, M.A. (2012). Cooperative breeding systems. — In: The evolution of parental care. Oxford University Press, Oxford, UK, p. 206-225.

Cant, M.A. & Field, J. (2005). Helping effort in a dominance hierarchy. — Behav. Ecol. 16, 708-715.

Cant, M.A., Hodge, S.J., Bell, M.B.V., Gilchrist, J.S. & Nichols, H.J. (2010). Reproductive control via eviction (but not the threat of eviction) in banded mongooses. — Proc. R. Soc. B: Biol. Sci. 277, 2219-2226.

Caro, T. (2005). Antipredator defenses in birds and mammals. — University of Chicago Press, Chicago, IL, USA.

Cervo, R., Dapporto, L., Beani, L., Strassmann, J.E. & Turillazzi, S. (2008). On status badges and quality signals in the paper wasp *Polistes dominulus*: body size, facial colour patterns and hierarchical rank. — Proc. R. Soc. B: Biol. Sci. 275, 1189-1196.

Chen, C.C. & Fernald, R.D. (2011). Visual information alone changes behavior and physiology during social interactions in a cichlid fish (*Astatotilapia burtoni*). — PLoS ONE 6.

Cho, H.J., Acharjee, S., Moon, M.J., Oh, D.Y., Vaudry, H., Kwon, H.B. & Seong, J.Y. (2007). Molecular evolution of neuropeptide receptors with regard to maintaining high affinity to their authentic ligands. — Gen. Comp. Endocrinol. 153, 98-107.

Christopher, L.P., Sarah, E.D.D., Bhumi, P. & Laura, M.H. (2021). Social experience interacts with serotonin to affect functional connectivity in the social behavior network following playback of social vocalizations in mice. — eNeuro 8, ENEURO.0247-20.2021.

Clayton, N.S. & Emery, N.J. (2007). The social life of corvids. — Curr. Biol. 17, R652-R656.

Cooper, M.A., Karom, M., Huhman, K.L. & Elliott Albers, H. (2005). Repeated agonistic encounters in hamsters modulate AVP V1a receptor binding. — Horm. Behav. 48, 545-551.

Copeland, D.L., Levay, B., Sivaraman, B., Beebe-Fugloni, C. & Earley, R.L. (2011). Metabolic costs of fighting are driven by contest performance in male convict cichlid fish. — Anim. Behav. 82, 271-280.

Cordoni, G. (2009). Social play in captive wolves (*Canis lupus*): not only an immature affair. — Behaviour 146, 1363-1385.

Creel, S. & Creel, N.M. (1995). Communal hunting and pack size in African wild dogs, *Lycaon pictus*. — Anim. Behav. 50, 1325-1339.

Cresswell, W. (1994). Flocking is an effective anti-predation strategy in redshanks, *Tringa totanus*. — Anim. Behav. 47, 433-442.

Cross, P.C., Lloyd-Smith, J.O., Bowers, J.A., Hay, C.T., Hofmeyr, M. & Getz, W.M. (2004). Integrating association data and disease dynamics in a social ungulate: bovine tuberculosis in African buffalo in the Kruger National Park. — Ann. Zool. Fenn., 879-892.

Culbert, B.M. & Balshine, S. (2019). Visual threat signals influence social interactions in a cooperatively breeding fish. — Anim. Behav. 151, 177-184.

Cunha, F.C.R.d., Fontenelle, J.C.R. & Griesser, M. (2017). Predation risk drives the expression of mobbing across bird species. — Behav. Ecol. 28, 1517-1523.

Curio, E. (1976). Prey selection. — In: The Ethology of Predation. Springer Berlin Heidelberg, Berlin, GER, p. 113-131.

Curio, E., Ernst, U. & Vieth, W. (1978). The adaptive significance of avian mobbing: II. Cultural transmission of enemy recognition in blackbirds: Effectiveness and some constraints. — Z. Tierpsychol. 48, 184-202.

Daly, M. (1978). The cost of mating. — Am. Nat. 112, 771-774.

De Dreu, C.K.W., Greer, L.L., Handgraaf, M.J.J., Shalvi, S., Van Kleef, G.A., Baas, M., Ten Velden, F.S., Van Dijk, E. & Feith, S.W.W. (2010). The neuropeptide oxytocin regulates parochial altruism in intergroup conflict among humans. — Science 328, 1408-1411.

De Silva, S., Ranjeewa, A.D.G. & Kryazhimskiy, S. (2011). The dynamics of social networks among female Asian elephants. — BMC Ecol. 11, 17.

De Vries, H., Stevens, J.M.G. & Vervaecke, H. (2006). Measuring and testing the steepness of dominance hierarchies. — Anim. Behav. 71, 585-592.

De Waal, F.B.M. (1986). The integration of dominance and social bonding in primates. — Q. Rev. Biol. 61, 459-479.

Deag, J.M. & Scott, G.W. (1999). “Conventional” signals in avian agonistic displays: integrating theory, data and different levels of analysis. — J. Theor. Biol. 196, 155-162.

Delville, Y., De Vries, G.J. & Ferris, C.F. (2000). Neural connections of the anterior hypothalamus and agonistic behavior in golden hamsters. — Brain Behav. Evol. 55, 53-76.

Desjardins, J.K., Fitzpatrick, J.L., Stiver, K.A., Van Der Kraak, G.J. & Balshine, S. (2008). Costs and benefits of polygyny in the cichlid *Neolamprologus pulcher*. — Anim. Behav. 75, 1771-1779.

Dewan, A.K., Maruska, K.P. & Tricas, T.C. (2008). Arginine vasotocin neuronal phenotypes among congeneric territorial and shoaling reef butterflyfishes: species, sex and reproductive season comparisons. — J. Neuroendocrinol. 20, 1382-1394.

Dewan, A.K., Ramey, M.L. & Tricas, T.C. (2011). Arginine vasotocin neuronal phenotypes, telencephalic fiber varicosities, and social behavior in butterflyfishes (Chaetodontidae): Potential similarities to birds and mammals. — Horm. Behav. 59, 56-66.

Dewan, A.K. & Tricas, T.C. (2011). Arginine vasotocin neuronal phenotypes and their relationship to aggressive behavior in the territorial monogamous multiband butterflyfish, *Chaetodon multicinctus*. — Brain Res. 1401, 74-84.

Dey, C.J., Dale, J. & Quinn, J.S. (2014). Manipulating the appearance of a badge of status causes changes in true badge expression. — Proc. R. Soc. Lond., Ser. B: Biol. Sci. 281, 20132680.

Dey, C.J., Reddon, A.R., O'connor, C.M. & Balshine, S. (2013). Network structure is related to social conflict in a cooperatively breeding fish. — Anim. Behav. 85, 395-402.

Dey, C.J., Tan, Q.J., O’connor, C.M., Reddon, A.R. & Caldwell, J.R. (2015). Dominance network structure across reproductive contexts in the cooperatively breeding cichlid fish *Neolamprologus pulcher*. — Curr. Zool. 61, 45-54.

Dierkes, P., Taborsky, M. & Kohler, U. (1999). Reproductive parasitism of broodcare helpers in a cooperatively breeding fish. — Behav. Ecol. 10, 510-515.

Donaldson, Z.R. & Young, L.J. (2008). Oxytocin, vasopressin, and the neurogenetics of sociality. — Science 322, 900-904.

Drews, C. (1993). The concept and definition of dominance in animal behaviour. — Behaviour 125, 283-313.

Dugatkin, L.A. & Earley, R.L. (2004). Individual recognition, dominance hierarchies and winner and loser effects. — Proc. R. Soc. Lond., Ser. B: Biol. Sci. 271, 1537-1540.

Earley, R.L. & Dugatkin, L.A. (2010). Behavior in groups. — In: Evolutionary Behavioral Ecology. Oxford University Press, Oxford, UK, p. 285-308.

Earley, R.L., Edwards, J.T., Aseem, O., Felton, K., Blumer, L.S., Karom, M. & Grober, M.S. (2006). Social interactions tune aggression and stress responsiveness in a territorial cichlid fish (*Archocentrus nigrofasciatus*). — Physiol. Behav. 88, 353-363.

Eaton, L. & Sloman, K.A. (2011). Subordinate brown trout exaggerate social signalling in turbid conditions. — Anim. Behav. 81, 603-608.

Eberhard, M.J.W. (1975). The evolution of social behavior by kin selection. — Q. Rev. Biol. 50, 1-33.

Ekman, J. (1987). Exposure and time use in willow tit flocks: the cost of subordination. — Anim. Behav. 35, 445-452.

English, S., Browning, L.E. & Raihani, N.J. (2015). Developmental plasticity and social specialization in cooperative societies. — Anim. Behav. 106, 37-42.

Enquist, M. & Leimar, O. (1990). The evolution of fatal fighting. — Anim. Behav. 39, 1-9.

Estevez, I., Andersen, I.-L. & Nævdal, E. (2007). Group size, density and social dynamics in farm animals. — Appl. Anim. Behav. Sci. 103, 185-204.

Fawcett, T.W., Marshall, J.A. & Higginson, A.D. (2015). The evolution of mechanisms underlying behaviour. — Curr. Zool. 61, 221-225.

Ferris, C.F., Axelson, J.F., Martin, A.M. & Roberge, L.F. (1989). Vasopressin immunoreactivity in the anterior hypothalamus is altered during the establishment of dominant/subordinate relationships between hamsters. — Neurosci. 29, 675-683.

Ferris, C.F. & Delville, Y. (1994). Vasopressin and serotonin interactions in the control of agonistic behavior. — Psychoneuroendocrinology 19, 593-601.

Filby, A.L., Paull, G.C., Hickmore, T.F.A. & Tyler, C.R. (2010). Unravelling the neurophysiological basis of aggression in a fish model. — BMC Genom. 11, 498.

Fischer, E.K., Westrick, S.E., Hartsough, L. & Hoke, K.L. (2018). Differences in neural activity, but not behavior, across social contexts in guppies, *Poecilia reticulata*. — Behav. Ecol. Sociobiol. 72, 131.

Fischer, S., Bohn, L., Oberhummer, E., Nyman, C. & Taborsky, B. (2017). Divergence of developmental trajectories is triggered interactively by early social and ecological experience in a cooperative breeder. — PNAS 114, E9300-E9307.

Fischer, S., Zöttl, M., Groenewoud, F. & Taborsky, B. (2014). Group-size-dependent punishment of idle subordinates in a cooperative breeder where helpers pay to stay. — Proc. R. Soc. Lond., Ser. B: Biol. Sci. 281, 20140184.

Fitzpatrick, J.L., Desjardins, J.K., Milligan, N., Stiver, K.A., Montgomerie, R. & Balshine, S. (2008). Female-mediated causes and consequences of status change in a social fish. — Proc. R. Soc. Lond., Ser. B: Biol. Sci. 275, 929-936.

Flack, J.C. & De Waal, F.B.M. (2004). Dominance style, social power, and conflict management: a conceptual framework. — In: Macaque societies: A model for the study of social organization. Cambridge University Press, Cambridge, UK, p. 157.

Flack, J.C. & De Waal, F.B.M. (2007). Context modulates signal meaning in primate communication. — PNAS 104, 1581-1586.

Forkman, B. & Haskell, M.J. (2004). The maintenance of stable dominance hierarchies and the pattern of aggression: support for the suppression hypothesis. — Ethology 110, 737-744.

Fox, J. & Weisberg, S. (2018). An R companion to applied regression. — Sage Publications, Thousand Oaks, CA, USA.

Fox, M.W. (1969). The anatomy of aggression and its ritualization in Canidae: a developmental and comparative study. — Behaviour, 242-258.

Freeberg, T.M., Dunbar, R.I.M. & Ord, T.J. (2012). Social complexity as a proximate and ultimate factor in communicative complexity. — Philos. Trans. R. Soc. Lond., Ser. B: Biol. Sci. 367, 1785-1801.

Friesen, C.N., Maclaine, K.D. & Hofmann, H.A. (2022). Social status mediates behavioral, endocrine, and neural responses to an intruder challenge in a social cichlid, *Astatotilapia burtoni*. — Horm. Behav. 145, 105241.

Frommen, J.G. (2020). Aggressive communication in aquatic environments. — Funct. Ecol. 34, 364-380.

Gager, Y. (2019). Information transfer about food as a reason for sociality in bats. — Mamm. Rev. 49, 113-120.

Garamszegi, L.Z., Rosivall, B., Hegyi, G., Szöllösi, E., Török, J. & Eens, M. (2006). Determinants of male territorial behavior in a Hungarian collared flycatcher population: plumage traits of residents and challengers. — Behav. Ecol. Sociobiol. 60, 663-671.

Gaston, A.J. (1978). The evolution of group territorial behavior and cooperative breeding. — Am. Nat. 112, 1091-1100.

Geist, V. (1966). The evolution of horn-like organs. — Behaviour 27, 175-214.

Geist, V. (1974). On the relationship of social evolution and ecology in ungulates. — Am. Zool. 14, 205-220.

Gerber, B., Stamer, A. & Stadtlander, T. (2015). Environmental enrichment and its effects on welfare in fish. — FiBL, Frick, Switzerland.

Ghahramani, Z.N., Perelmuter, J.T., Varughese, J., Kyaw, P., Palmer, W.C., Sisneros, J.A. & Forlano, P.M. (2022). Activation of noradrenergic locus coeruleus and social behavior network nuclei varies with duration of male midshipman advertisement calls. — Behav. Brain Res. 423, 113745.

Gibson, R.N. (1967). The agonistic behaviour of juvenile *Blennius pholis* L.(Teleostei). — Behaviour 30, 192-217.

Godfrey, S.S., Sih, A. & Bull, C.M. (2013). The response of a sleepy lizard social network to altered ecological conditions. — Anim. Behav. 86, 763-772.

Godwin, J.C. & Thompson, R.R. (2012). Nonapeptides and social behavior in fishes. — Horm. Behav. 61, 230-238.

Goodale, E., Beauchamp, G., Magrath, R.D., Nieh, J.C. & Ruxton, G.D. (2010). Interspecific information transfer influences animal community structure. — Trends Ecol. Evol. 25, 354-361.

Goodson, J.L. (2005). The vertebrate social behavior network: Evolutionary themes and variations. — Horm. Behav. 48, 11-22.

Goodson, J.L. (2013). Deconstructing sociality, social evolution and relevant nonapeptide functions. — Psychoneuroendocrinology 38, 465-478.

Goodson, J.L. & Bass, A.H. (2001). Social behavior functions and related anatomical characteristics of vasotocin/vasopressin systems in vertebrates. — Brain Res. Rev. 35, 246-265.

Goodson, J.L., Evans, A.K. & Bass, A.H. (2003). Putative isotocin distributions in sonic fish: Relation to vasotocin and vocal–acoustic circuitry. — J. Comp. Neurol. 462, 1-14.

Goodson, J.L., Evans, A.K. & Soma, K.K. (2005). Neural responses to aggressive challenge correlate with behavior in nonbreeding sparrows. — Neuroreport 16, 1719-1723.

Goodson, J.L. & Kabelik, D. (2009). Dynamic limbic networks and social diversity in vertebrates: From neural context to neuromodulatory patterning. — Front. Neuroendocrinol. 30, 429-441.

Goodson, J.L., Kabelik, D. & Schrock, S.E. (2009). Dynamic neuromodulation of aggression by vasotocin: influence of social context and social phenotype in territorial songbirds. — Biol. Lett. 5, 554-556.

Goodson, J.L. & Thompson, R.R. (2010). Nonapeptide mechanisms of social cognition, behavior and species-specific social systems. — Curr. Opin. Neurobiol. 20, 784-794.

Goossens, N., Blähser, S., Oksche, A., Vandesande, F. & Dierickx, K. (1977). Immunocytochemical investigation of the hypothalamo-neurohypophysial system in birds. — Cell Tissue Res. 184, 1-13.

Gouzènes, L., Desarménien, M.G., Hussy, N., Richard, P. & Moos, F.C. (1998). Vasopressin regularizes the phasic firing pattern of rat hypothalamic magnocellular vasopressin neurons. — J. Neurosci. 18, 1879-1885.

Grantner, A. & Taborsky, M. (1998). The metabolic rates associated with resting, and with the performance of agonistic, submissive and digging behaviours in the cichlid fish *Neolamprologus pulcher* (Pisces: Cichlidae). — J. Comp. Physiol., B 168, 427-433.

Graw, B. & Manser, M.B. (2007). The function of mobbing in cooperative meerkats. — Anim. Behav. 74, 507-517.

Gray, J.A. (1971). Sex differences in emotional behaviour in mammals including man: endocrine bases. — Acta Psychol. 35, 29-46.

Greenwood, A.K., Wark, A.R., Fernald, R.D. & Hofmann, H.A. (2008). Expression of arginine vasotocin in distinct preoptic regions is associated with dominant and subordinate behaviour in an African cichlid fish. — Proc. R. Soc. Lond., Ser. B: Biol. Sci. 275, 2393-2402.

Grober, M.S., George, A.A., Watkins, K.K., Carneiro, L.A. & Oliveira, R.F. (2002). Forebrain AVT and courtship in a fish with male alternative reproductive tactics. — Brain Res. Bull. 57, 423-425.

Groenewoud, F., Frommen, J.G., Josi, D., Tanaka, H., Jungwirth, A. & Taborsky, M. (2016). Predation risk drives social complexity in cooperative breeders. — Proc. Natl. Acad. Sci. USA 113, 4104-4109.

Gross, M.R. & Macmillan, A.M. (1981). Predation and the evolution of colonial nesting in bluegill sunfish (*Lepomis macrochirus*). — Behav. Ecol. Sociobiol. 8, 163-174.

Guhl, A. (1958). The development of social organisation in the domestic chick. — Anim. Behav. 6, 92-111.

Guilford, T. & Dawkins, M.S. (1995). What are conventional signals? — Anim. Behav. 49, 1689-1695.

Hamilton, I.M., Heg, D. & Nicole, B. (2005). Size differences within a dominance hierarchy influence conflict and help in a cooperatively breeding cichlid. — Behaviour 142, 1591-1613.

Hamilton, W.D. (1964). The genetical evolution of social behaviour. II. — J. Theor. Biol. 7, 17-52.

Hardy, I.C.W. & Briffa, M. (2013). Animal contests. — Cambridge University Press, Cambridge, UK.

Hattori, T. & Wilczynski, W. (2009). Comparison of arginine vasotocin immunoreactivity differences in dominant and subordinate green anole lizards. — Physiol. Behav. 96, 104-107.

Hawk, J. & Abel, T. (2017). Role of gene transcription in long-term memory storage. — In: Encyclopedia of Behavioral Neuroscience, 2nd edition. Elsevier, Oxford, UK, p. 405-426.

Heg, D., Bachar, Z., Brouwer, L. & Taborsky, M. (2004). Predation risk is an ecological constraint for helper dispersal in a cooperatively breeding cichlid. — Proc. R. Soc. Lond., Ser. B: Biol. Sci. 271, 2367-2374.

Heg, D., Brouwer, L., Bachar, Z. & Taborsky, M. (2005). Large group size yields group stability in the cooperatively breeding cichlid *Neolamprologus pulcher*. — Behaviour 142, 1615-1641.

Heg, D., Schürch, R. & Rothenberger, S. (2011). Behavioral type and growth rate in a cichlid fish. — Behav. Ecol. 22, 1227-1233.

Hellmann, J.K., Ligocki, I.Y., O'connor, C.M., Reddon, A.R., Farmer, T.M., Marsh-Rollo, S.E., Balshine, S. & Hamilton, I.M. (2016). The influence of status and the social environment on energy stores in a social fish. — J. Fish Biol. 88, 1321-1334.

Hellmann, J.K., Reddon, A.R., Ligocki, I.Y., O'connor, C.M., Garvy, K.A., Marsh-Rollo, S.E., Hamilton, I.M. & Balshine, S. (2015). Group response to social perturbation: impacts of isotocin and the social landscape. — Anim. Behav. 105, 55-62.

Henzi, S.P., Lusseau, D., Weingrill, T., Van Schaik, C.P. & Barrett, L. (2009). Cyclicity in the structure of female baboon social networks. — Behav. Ecol. Sociobiol. 63, 1015-1021.

Herbert, J. (1977). Hormones and behaviour. — Proc. R. Soc. Lond., Ser. B: Biol. Sci. 199, 425-443.

Hick, K., Reddon, A.R., O'connor, C.M. & Balshine, S. (2014). Strategic and tactical fighting decisions in cichlid fishes with divergent social systems. — Behaviour 151, 47-71.

Ho, J.M., Murray, J.H., Demas, G.E. & Goodson, J.L. (2010). Vasopressin cell groups exhibit strongly divergent responses to copulation and male–male interactions in mice. — Horm. Behav. 58, 368-377.

Hoelzer, G. (1987). The effect of early experience on aggression in two territorial scorpaenid fishes. — Environ. Biol. Fishes 19, 183-194.

Hoglund, E., Balm, P.H. & Winberg, S. (2000). Skin darkening, a potential social signal in subordinate arctic charr (*Salvelinus alpinus*): the regulatory role of brain monoamines and pro-opiomelanocortin-derived peptides. — J. Exp. Biol. 203, 1711-1721.

Höglund, J. & Alatalo, R.V. (1995). Leks. — Princeton University Press, Princeton, NJ, USA.

Hoi, H., Darolova, A., König, C. & Kristofík, J. (1998). The relation between colony size, breeding density and ectoparasite loads of adult European bee-eaters (*Merops apiaster*). — Ecoscience 5, 156-163.

Hollén, L.I., Bell, M.B. & Radford, A.N. (2008). Cooperative sentinel calling? Foragers gain increased biomass intake. — Curr. Biol. 18, 576-579.

Huffman, L.S., Hinz, F.I., Wojcik, S., Aubin-Horth, N. & Hofmann, H.A. (2015). Arginine vasotocin regulates social ascent in the African cichlid fish *Astatotilapia burtoni*. — Gen. Comp. Endocrinol. 212, 106-113.

Huffman, L.S., O’connell, L.A., Kenkel, C.D., Kline, R.J., Khan, I.A. & Hofmann, H.A. (2012). Distribution of nonapeptide systems in the forebrain of an African cichlid fish, *Astatotilapia burtoni*. — J. Chem. Neuroanat. 44, 86-97.

Huntingford, F.A. & Turner, A.K. (2013). Animal conflict. — Springer Science & Business Media, London, UK.

Huntingford, F.A., Turner, A.K. & Downie, L.M. (1987). The consequences of animal conflict. — In: Animal Conflict. Springer, Dordrecht, NL, p. 227-250.

Hurd, P.L. (1997). Cooperative signalling between opponents in fish fights. — Anim. Behav. 54, 1309-1315.

Insel, T.R. (2010). The challenge of translation in social neuroscience: a review of oxytocin, vasopressin, and affiliative behavior. — Neuron 65, 768-779.

Insel, T.R. & Young, L.J. (2000). Neuropeptides and the evolution of social behavior. — Curr. Opin. Neurobiol. 10, 784-789.

Issa, F.A. & Edwards, D.H. (2006). Ritualized submission and the reduction of aggression in an invertebrate. — Curr. Biol. 16, 2217-2221.

Iwata, E., Nagai, Y. & Sasaki, H. (2010). Immunohistochemistry of brain arginine vasotocin and isotocin in false clown anemonefish *Amphiprion ocellaris*. —Open Fish Sci. J. 3.

Jennings, D.J., Gammell, M.P., Carlin, C.M. & Hayden, T.J. (2002). Does lateral presentation of the palmate antlers during fights by fallow deer (*Dama dama* L.) signify dominance or submission? — Ethology 108, 389-401.

Jennions, M.D. & Macdonald, D.W. (1994). Cooperative breeding in mammals. — Trends Ecol. Evol. 9, 89-93.

Johnson, Z.V. & Young, L.J. (2017). Oxytocin and vasopressin neural networks: Implications for social behavioral diversity and translational neuroscience. — Neurosci. Biobehav. Rev. 76, 87-98.

Johnsson, J.I., Brockmark, S. & Näslund, J. (2014). Environmental effects on behavioural development consequences for fitness of captive-reared fishes in the wild. — J. Fish Biol. 85, 1946-1971.

Judge, P.G. & De Waal, F.B.M. (1993). Conflict avoidance among rhesus monkeys: coping with short-term crowding. — Anim. Behav. 46, 221-232.

Kabelik, D., Alix, V.C., Burford, E.R. & Singh, L.J. (2013). Aggression- and sex-induced neural activity across vasotocin populations in the brown anole. — Horm. Behav. 63, 437-446.

Kabelik, D., Weitekamp, C.A., Choudhury, S.C., Hartline, J.T., Smith, A.N. & Hofmann, H.A. (2018). Neural activity in the social decision-making network of the brown anole during reproductive and agonistic encounters. — Horm. Behav. 106, 178-188.

Kadry, V.O. & Barreto, R.E. (2010). Environmental enrichment reduces aggression of pearl cichlid, *Geophagus brasiliensis*, during resident-intruder interactions. — Neotrop. Ichthyol. 8.

Kasper, C., Vierbuchen, M., Ernst, U., Fischer, S., Radersma, R., Raulo, A., Cunha-Saraiva, F., Wu, M., Mobley, K.B. & Taborsky, B. (2017). Genetics and developmental biology of cooperation. — Mol. Ecol. 26, 4364-4377.

Kaufmann, J.H. (1983). On the definitions and functions of dominance and territoriality. — Biol. Rev. 58, 1-20.

Keenleyside, M.H.A. & Yamamoto, F.T. (1962). Territorial behaviour of juvenile Atlantic salmon (*Salmo salar* L.). — Behaviour 19, 139-168.

Kelly, A.M. (2022). A consideration of brain networks modulating social behavior. — Horm. Behav. 141, 105138.

Kemp, P.S., Armstrong, J.D. & Gilvear, D.J. (2005). Behavioural responses of juvenile Atlantic salmon (*Salmo salar*) to presence of boulders. — River Res. Appl. 21, 1053-1060.

King, J.A. (1973). The ecology of aggressive behavior. — Annu. Rev. Ecol. Syst. 4, 117-138.

Kleszczyńska, A., Sokołowska, E. & Kulczykowska, E. (2012). Variation in brain arginine vasotocin (AVT) and isotocin (IT) levels with reproductive stage and social status in males of three-spined stickleback (*Gasterosteus aculeatus*). — Gen. Comp. Endocrinol. 175, 290-296.

Kochhann, D. & Val, A.L. (2017). Social hierarchy and resting metabolic rate in the dwarf cichlid *Apistogramma agassizii*: the role of habitat enrichment. — Hydrobiologia 789, 123-131.

Kokko, H., Johnstone, R.A. & Clutton-Brock, T.H. (2001). The evolution of cooperative breeding through group augmentation. — Proc. R. Soc. Lond., Ser. B: Biol. Sci. 268, 187-196.

Kollack-Walker, S. & Newman, S.W. (1995). Mating and agonistic behavior produce different patterns of Fos immunolabeling in the male Syrian hamster brain. — Neuroscience 66, 721-736.

Krause, J. & Ruxton, G.D. (2002). Living in groups. — Oxford University Press, Cary, NC, USA.

Kress, S. & Wullimann, M.F. (2012). Correlated basal expression of immediate early gene egr1 and tyrosine hydroxylase in zebrafish brain and downregulation in olfactory bulb after transitory olfactory deprivation. — J. Chem. Neuroanat. 46, 51-66.

Kulczykowska, E. (2008). Arginine vasotocin and isotocin as multifunctional hormones, neurotransmitters and neuromodulators in fish. — In: Avances en Endocrinología Comparada. Servicio de Publicaciones, Universidad de Cádiz, Cádiz, Spain, p. 41–47.

Kulczykowska, E. & Kleszczyńska, A. (2014). Brain arginine vasotocin and isotocin in breeding female three-spined sticklebacks (*Gasterosteus aculeatus*): The presence of male and egg deposition. — Gen. Comp. Endocrinol. 204, 8-12.

Kutsukake, N. & Clutton-Brock, T.H. (2006). Aggression and submission reflect reproductive conflict between females in cooperatively breeding meerkats *Suricata suricatta*. — Behav. Ecol. Sociobiol. 59, 541-548.

Kutsukake, N. & Clutton-Brock, T.H. (2008). Do meerkats engage in conflict management following aggression? Reconciliation, submission and avoidance. — Anim. Behav. 75, 1441-1453.

Kuznetsova, A., Brockhoff, P.B. & Christensen, R.H.B. (2017). lmerTest package: tests in linear mixed effects models. — J. Stat. Softw. 82, 1 - 26.

Kvarnemo, C. (1998). Temperature modulates competitive behaviour: why sand goby males fight more in warmer water. — Ethol. Ecol. Evol. 10, 105-114.

Lachance, A.-A., Dutil, J.-D., Larocque, R. & Daigle, G. (2010). Shelter use and behaviour of juvenile Spotted Wolffish (*Anarhichas minor*) in an experimental context. — Environ. Biol. Fishes 88, 207-215.

Lane, S.M. & Briffa, M. (2017). The price of attack: rethinking damage costs in animal contests. — Anim. Behav. 126, 23-29.

Larson, E.T., O'malley, D.M. & Melloni, R.H. (2006). Aggression and vasotocin are associated with dominant–subordinate relationships in zebrafish. — Behav. Brain Res. 167, 94-102.

Le Vin, A., Mable, B. & Arnold, K. (2010). Kin recognition via phenotype matching in a cooperatively breeding cichlid, *Neolamprologus pulcher*. — Anim. Behav. 79, 1109-1114.

Le Vin, A.L., Mable, B., Taborsky, M., Heg, D. & Arnold, K.E. (2011). Individual variation in helping in a cooperative breeder: relatedness versus behavioural type. — Anim. Behav. 82, 467-477.

Lee, H.-J., Macbeth, A.H., Pagani, J.H. & Young 3rd, W.S. (2009). Oxytocin: the great facilitator of life. — Prog. Neurobiol. 88, 127-151.

Lee, J.S.F. & Berejikian, B.A. (2009). Structural complexity in relation to the habitat preferences, territoriality, and hatchery rearing of juvenile China rockfish (*Sebastes nebulosus*). — Environ. Biol. Fishes 84, 411-419.

Lehmann, L., Keller, L., West, S. & Roze, D. (2007). Group selection and kin selection: Two concepts but one process. — PNAS 104, 6736-6739.

Lema, S., Sanders, K. & Walti, K. (2015). Arginine vasotocin, isotocin and nonapeptide receptor gene rxpression link to social status and aggression in sex-dependent patterns. — J. Neuroendocrinol. 27, 142-157.

Lema, S.C. (2006). Population divergence in plasticity of the AVT system and its association with aggressive behaviors in a Death Valley pupfish. — Horm. Behav. 50, 183-193.

Leyhausen, P. & Tonkin, B.A. (1979). Cat behaviour. The predatory and social behaviour of domestic and wild cats. — Garland STPM Press., New York City, NY, USA.

Ligon, R.A. (2014). Defeated chameleons darken dynamically during dyadic disputes to decrease danger from dominants. — Behav. Ecol. Sociobiol. 68, 1007-1017.

Liley, N. & Stacey, N. (1983). Hormones, pheromones, and reproductive behavior in fish. — In: Fish Physiology, Academic Press Inc., New York City, NY, USA, p. 1-63.

Lima, S.L. (1995). Back to the basics of anti-predatory vigilance: the group-size effect. — Anim. Behav. 49, 11-20.

Long, K.D. & Salbaum, J.M. (1998). Evolutionary conservation of the immediate-early gene ZENK. — Mol. Biol. Evol. 15, 284-292.

Lorenz, K. (1943). Die angeborenen formen möglicher erfahrung. — Z. Tierpsychol. 5, 235-409.

Lorenz, K. (1966). On aggression. — Routledge, London, UK.

Loveland, J.L. & Fernald, R.D. (2017). Differential activation of vasotocin neurons in contexts that elicit aggression and courtship. — Behav. Brain Res. 317, 188-203.

Lüdecke, D., Ben-Shachar, M.S., Patil, I., Waggoner, P. & Makowski, D. (2021). performance: An R package for assessment, comparison and testing of statistical models. — JOSS 6, 3139.

Lukas, D. & Clutton-Brock, T. (2012). Life histories and the evolution of cooperative breeding in mammals. — Proc. R. Soc. B: Biol. Sci. 279, 4065-4070.

Maan, M.E., Groothuis, T.G.G. & Wittenberg, J. (2001). Escalated fighting despite predictors of conflict outcome: solving the paradox in a South American cichlid fish. — Anim. Behav. 62, 623-634.

Macdonald, D.W. (1983). The ecology of carnivore social behaviour. — Nature 301, 379.

Maestripieri, D. & Wallen, K. (1997). Affiliative and submissive communication in rhesus macaques. — Primates 38, 127-138.

Magnuson, J.J. (1962). An analysis of aggressive behavior, growth, and competition for food and space in medaka (*Oryzias latipes* (pisces, cyprinodontidae)). — Can. J. Zool. 40, 313-363.

Magurran, A.E. & Pitcher, T.J. (1983). Foraging, timidity and shoal size in minnows and goldfish. — Behav. Ecol. Sociobiol. 12, 147-152.

Majolo, B., De Bortoli Vizioli, A. & Schino, G. (2008). Costs and benefits of group living in primates: group size effects on behaviour and demography. — Anim. Behav. 76, 1235-1247.

Majolo, B. & Huang, P. (2022). Group Living. — In: Encyclopedia of Animal Cognition and Behavior. Springer International Publishing, Cham, Switzerland, p. 3021-3032.

Manara, V., Ruberto, T., Swaney, W.T. & Reddon, A.R. (2023). Subordinate submissive responses are predicted by dominant behaviour in a cooperatively breeding fish. — Behaviour 160, 1-18.

Marsh, K.E., Creutz, L.M., Hawkins, M.B. & Godwin, J. (2006). Aromatase immunoreactivity in the bluehead wrasse brain, *Thalassoma bifasciatum*: Immunolocalization and co-regionalization with arginine vasotocin and tyrosine hydroxylase. — Brain Res. 1126, 91-101.

Maruska, K.P., Anselmo, C.M., King, T., Mobley, R.B., Ray, E.J. & Wayne, R. (2022). Endocrine and neuroendocrine regulation of social status in cichlid fishes. — Horm. Behav. 139, 105110.

Matsumura, S. & Hayden, T.J. (2006). When should signals of submission be given?–A game theory model. — J. Theor. Biol. 240, 425-433.

Maynard Smith, J. (1982). Evolution and the theory of games. — Cambridge University Press, Cambridge, UK.

Maynard Smith, J. & Harper, D. (2003). Animal signals. — Oxford University Press, Oxford, UK.

Maynard Smith, J. & Parker, G.A. (1976). The logic of asymmetric contests. — Anim. Behav. 24, 159-175.

Maynard Smith, J. & Price, G.R. (1973). The logic of animal conflict. — Nature 246, 15-18.

Mech, D. & Boitani, L. (2003). Wolves: behaviour, ecology, and conservation. — The University of Chicago Press, Chicago, IL, USA.

Moore, F.L., Boyd, S.K. & Kelley, D.B. (2005). Historical perspective: hormonal regulation of behaviors in amphibians. — Horm. Behav. 48, 373-383.

Moore, F.L. & Lowry, C.A. (1998). Comparative neuroanatomy of vasotocin and vasopressin in amphibians and other vertebrates. — Comp. Biochem. Physiol., C: Toxicol. Pharmacol. 119, 251-260.

Morrell, L.J., Lindström, J. & Ruxton, G.D. (2005). Why are small males aggressive? — Proc. R. Soc. Lond., Ser. B: Biol. Sci. 272, 1235-1241.

Moyle, P.B. & Cech, J.J. (1996). Fishes: an introduction to ichthyology. — Pearson Prentice Hall, Upper Saddle River, NJ, USA.

Mulder, R.A. & Langmore, N.E. (1993). Dominant males punish helpers for temporary defection in superb fairy-wrens. — Anim. Behav. 45, 830-833.

Näslund, J. & Johnsson, J.I. (2016). Environmental enrichment for fish in captive environments: effects of physical structures and substrates. — Fish Fish. 17, 1-30.

Neat, F.C., Taylor, A.C. & Huntingford, F.A. (1998). Proximate costs of fighting in male cichlid fish: the role of injuries and energy metabolism. — Anim. Behav. 55, 875.

Nelson, R.J. (2006). Biology of Aggression. — Oxford University Press., New York City, NY, USA.

Neumann, C., Duboscq, J., Dubuc, C., Ginting, A., Irwan, A.M., Agil, M., Widdig, A. & Engelhardt, A. (2011). Assessing dominance hierarchies: validation and advantages of progressive evaluation with Elo-rating. — Anim. Behav. 82, 911-921.

Newman, S.W. (1999). The medial extended amygdala in male reproductive behavior. A node in the mammalian social behavior network. — Ann. N. Y. Acad. Sci. 877, 242-257.

Norman, K., Pellis, S.M., Barrett, L. & Henzi, S.P. (2015). Down but not out: Supine postures as facilitators of play in domestic dogs. — Behav. Processes 110, 88-95.

O'connell, L.A. & Hofmann, H.A. (2011). The vertebrate mesolimbic reward system and social behavior network: A comparative synthesis. — J. Comp. Neurol. 519, 3599-3639.

O'connor, K.I., Metcalfe, N.B. & Taylor, A.C. (1999). Does darkening signal submission in territorial contests between juvenile Atlantic salmon, *Salmo salar*? — Anim. Behav. 58, 1269-1276.

O'connor, K.I., Metcalfe, N.B. & Taylor, A.C. (2000). The effects of prior residence on behavior and growth rates in juvenile Atlantic salmon (*Salmo salar*). — Behav. Ecol. 11, 13-18.

O’connell, L.A. & Hofmann, H.A. (2011). Genes, hormones, and circuits: An integrative approach to study the evolution of social behavior. — Front. Neuroendocrinol. 32, 320-335.

Oliveira, R.F. (2004). Social modulation of androgens in vertebrates: mechanisms and function. — In: Advances in the Study of Behavior. Elsevier Academic Press, Oxford, UK, p. 165-240.

Oliveira, R.F. (2009). Social behavior in context: Hormonal modulation of behavioral plasticity and social competence. — Integr. Comp. Biol. 49, 423-440.

Ota, Y., Ando, H., Ueda, H. & Urano, A. (1999). Seasonal changes in expression of neurohypophysial hormone genes in the preoptic nucleus of immature female masu salmon. — Gen. Comp. Endocrinol. 116, 31-39.

Packer, C. & Ruttan, L. (1988). The evolution of cooperative hunting. — Am. Nat. 132, 159-198.

Parker, G.A. (1974). Assessment strategy and the evolution of fighting behaviour. — J. Theor. Biol. 47, 223-243.

Partridge, B.L. (1982). The structure and function of fish schools. — Sci. Am. 246, 114-123.

Pellis, S.M. & Pellis, V.C. (2015). Are agonistic behavior patterns signals or combat tactics—Or does it matter? Targets as organizing principles of fighting. — Physiol. Behav. 146, 73-78.

Perrone, R. & Silva, A.C. (2018). Status-sependent vasotocin modulation of dominance and subordination in the weakly electric fish *Gymnotus omarorum*. — Front. Behav. Neurosci. 12, 1.

Petit, O. (2010). Social competition and conflict resolution. — In: Encyclopedia of Behavioral Neuroscience. Elsevier, Amsterdam, NL, p. 276–281.

Petren, K. & Case, T.J. (1998). Habitat structure determines competition intensity and invasion success in gecko lizards. — PNAS 95, 11739-11744.

Pinter-Wollman, N., Hobson, E.A., Smith, J.E., Edelman, A.J., Shizuka, D., De Silva, S., Waters, J.S., Prager, S.D., Sasaki, T. & Wittemyer, G. (2014). The dynamics of animal social networks: analytical, conceptual, and theoretical advances. — Behav. Ecol. 25, 242-255.

Pouso, P., Cabana, Á., Francia, V. & Silva, A. (2023). Involvement of neuropeptidergic neurons in the establishment of dominance in a teleost model of non-breeding aggression: neuropeptide-specific and status-dependent actions. —Research Square.

Pouso, P., Cabana, Á., Goodson, J.L. & Silva, A. (2019). Preoptic area activation and vasotocin involvement in the reproductive behavior of a weakly pulse-type electric fish, *Brachyhypopomus gauderio*. — Frontiers in Integrative Neuroscience 13.

Qiao, X., Yan, Y., Wu, R., Tai, F., Hao, P., Cao, Y. & Wang, J. (2014). Sociality and oxytocin and vasopressin in the brain of male and female dominant and subordinate mandarin voles. — J. Comp. Physiol. A 200, 149-159.

R-Core-Team. (2023). R: A language and environment for statistical computing. — In. R Foundation for Statistical Computing, Vienna, AT. Retrieved from https://www.R-project.org/.

Ramallo, M.R., Grober, M., Cánepa, M.M., Morandini, L. & Pandolfi, M. (2012). Arginine-vasotocin expression and participation in reproduction and social behavior in males of the cichlid fish *Cichlasoma dimerus*. — Gen. Comp. Endocrinol. 179, 221-231.

Reddon, A.R., Balk, D. & Balshine, S. (2013). Probing aggressive motivation during territorial contests in a group-living cichlid fish. — Behav. Processes 92, 47-51.

Reddon, A.R., Dey, C.J. & Balshine, S. (2019). Submissive behaviour is mediated by sex, social status, relative body size and shelter availability in a social fish. — Anim. Behav. 155, 131-139.

Reddon, A.R., O'connor, C.M., Marsh-Rollo, S.E. & Balshine, S. (2012). Effects of isotocin on social responses in a cooperatively breeding fish. — Anim. Behav. 84, 753-760.

Reddon, A.R., O'connor, C.M., Marsh-Rollo, S.E., Balshine, S., Gozdowska, M. & Kulczykowska, E. (2015). Brain nonapeptide levels are related to social status and affiliative behaviour in a cooperatively breeding cichlid fish. — R. Soc. Open Sci. 2, 140072.

Reddon, A.R., O'connor, C.M., Nesjan, E., Cameron, J., Hellmann, J.K., Ligocki, I.Y., Marsh-Rollo, S.E., Hamilton, I.M., Wylie, D.R. & Hurd, P.L. (2017). Isotocin neuronal phenotypes differ among social systems in cichlid fishes. — R. Soc. Open Sci. 4, 170350.

Reddon, A.R., Ruberto, T. & Reader, S.M. (2022). Submission signals in animal groups. — Behaviour 159, 1-20.

Reddon, A.R., Voisin, M.R., Menon, N., Marsh-Rollo, S.E., Wong, M.Y.L. & Balshine, S. (2011). Rules of engagement for resource contests in a social fish. — Anim. Behav. 82, 93-99.

Reyes-Contreras, M., Santiago, C. & Taborsky, B. (2023). Behavioural profiles in a wild population of a cooperatively breeding cichlid. — Ethology, 1-15.

Richer-De-Forges, M.M., Crampton, W.G.R. & Albert, J.S. (2009). A new species of *Gymnotus* (Gymnotiformes, Gymnotidae) from Uruguay: description of a model species in neurophysiological research. — Copeia 2009, 538-544.

Riebli, T., Avgan, B., Bottini, A.-M., Duc, C., Taborsky, M. & Heg, D. (2011). Behavioural type affects dominance and growth in staged encounters of cooperatively breeding cichlids. — Anim. Behav. 81, 313-323.

Rowell, T.E. (1974). The concept of social dominance. — Behav. Biol. 11, 131-154.

Ruberto, T., Talbot, J.L. & Reddon, A.R. (2020). Head up displays are a submission signal in the group-living daffodil cichlid. — Behav. Processes 181, 104271.

Saito, D., Komatsuda, M. & Urano, A. (2004). Functional organization of preoptic vasotocin and isotocin neurons in the brain of rainbow trout: central and neurohypophysial projections of single neurons. — Neuroscience 124, 973-984.

Sánchez-Hernández, P., Ramírez-Pinilla, M.P. & Molina-Borja, M. (2012). Agonistic and courtship behaviour patterns in the skink *Chalcides viridanus* (Fam. Scincidae) from Tenerife. — Acta Ethol. 15, 65-71.

Sánchez-Tójar, A., Schroeder, J. & Farine, D.R. (2018). A practical guide for inferring reliable dominance hierarchies and estimating their uncertainty. — J. Anim. Ecol. 87, 594-608.

Sapolsky, R.M. (1982). The endocrine stress-response and social status in the wild baboon. — Horm. Behav. 16, 279-292.

Sapolsky, R.M. (2005). The influence of social hierarchy on primate health. — Science 308, 648-652.

Schenkel, R. (1967). Submission: its features and function in the wolf and dog. — Am. Zool. 7, 319-329.

Schjelderup-Ebbe, T. (1922). Beiträge zur sozialpsychologie des haushuhns. — Z. Psychol. Physiol. Si. 88, 225–252.

Schmid-Hempel, P. (2017). Parasites and their social hosts. — Trends Parasitol. 33, 453-462.

Semsar, K., Kandel, F.L.M. & Godwin, J. (2001). Manipulations of the AVT system shift social status and related courtship and aggressive behavior in the bluehead wrasse. — Horm. Behav. 40, 21-31.

Seppänen, J.-T., Forsman, J.T., Mönkkönen, M. & Thomson, R.L. (2007). Social information use is a process across time, space, and ecology, reaching heterospecifics. — Ecology 88, 1622-1633.

Shea, S.A., Fedynich, A.M. & Wester, D.B. (2021). Assessment of the helminth fauna in northern bobwhites (*Colinus virginianus*) occurring within South Texas. — J. Helminthol. 95, e5.

Shen, S.F., Emlen, S.T., Koenig, W.D. & Rubenstein, D.R. (2017). The ecology of cooperative breeding behaviour. — Ecol. Lett. 20, 708-720.

Silk, J.B. (2007). Animal behavior: conflict management is for the birds. — Curr. Biol. 17, R50-R51.

Silva, A.C., Perrone, R., Zubizarreta, L., Batista, G. & Stoddard, P.K. (2013). Neuromodulation of the agonistic behavior in two species of weakly electric fish that display different types of aggression. — J. Exp. Biol. 216, 2412-2420.

Smith-Aguilar, S.E., Ramos-Fernández, G. & Getz, W.M. (2016). Seasonal Changes in Socio-Spatial Structure in a Group of Free-Living Spider Monkeys (*Ateles geoffroyi*). — PLoS ONE 11, e0157228.

Snell, H.L., Jennings, R.D., Snell, H.M. & Harcourt, S. (1988). Intrapopulation variation in predator-avoidance performance of Galápagos lava lizards: The interaction of sexual and natural selection. — Evol. Ecol. 2, 353-369.

Soares, M.C., Bshary, R., Fusani, L., Goymann, W., Hau, M., Hirschenhauser, K. & Oliveira, R.F. (2010). Hormonal mechanisms of cooperative behaviour. — Philos. Trans. R. Soc. Lond., Ser. B: Biol. Sci. 365, 2737-2750.

Sopinka, N.M., Fitzpatrick, J.L., Desjardins, J.K., Stiver, K.A., Marsh-Rollo, S.E. & Balshine, S. (2009). Liver size reveals social status in the African cichlid *Neolamprologus pulcher*. — J. Fish Biol. 75, 1-16.

Stander, P.E. (1992). Cooperative hunting in lions: the role of the individual. — Behav. Ecol. Sociobiol. 29, 445-454.

Stenberg, M. & Persson, A. (2005). The effects of spatial food distribution and group size on foraging behaviour in a benthic fish. — Behav. Processes 70, 41-50.

Stensland, E., Angerbjörn, A. & Berggren, P. (2003). Mixed species groups in mammals. — Mamm. Rev. 33, 205-223.

Stiver, K.A., Dierkes, P., Taborsky, M. & Balshine, S. (2004). Dispersal patterns and status change in a co-operatively breeding cichlid *Neolamprologus pulcher*: evidence from microsatellite analyses and behavioural observations. — J. Fish Biol. 65, 91-105.

Subhedar, N., Deshmukh, M.K., Jain, M.R., Khan, F.A. & Krishna, N.S.R. (2008). Activation of hypothalamic neurons by intraovarian pressure signals in a teleost fish, *Clarias batrachus*: Role of mechanosensitive channels. — Brain Behav. Evol. 47, 179-184.

Summers, C.H. & Winberg, S. (2006). Interactions between the neural regulation of stress and aggression. — J. Exp. Biol. 209, 4581-4589.

Sumpter, D.J. (2010). Collective animal behavior. — Princeton University Press, Princeton, NJ, USA.

Suter, H.C. & Huntingford, F.A. (2002). Eye colour in juvenile Atlantic salmon: effects of social status, aggression and foraging success. — J. Fish Biol. 61, 606-614.

Taborsky, B., Arnold, C., Junker, J. & Tschopp, A. (2012). The early social environment affects social competence in a cooperative breeder. — Anim. Behav. 83, 1067-1074.

Taborsky, M. (1984). Broodcare helpers in the cichlid fish *Lamprologus brichardi*: their costs and benefits. — Anim. Behav. 32, 1236-1252.

Taborsky, M. (1985). Breeder-helper conflict in a cichlid fish with broodcare helpers: an experimental analysis. — Behaviour 95, 45-75.

Taborsky, M. (2014). Tribute to Tinbergen: the four problems of biology. A critical appraisal. — Ethology 120, 224-227.

Taborsky, M. & Grantner, A. (1998). Behavioural time–energy budgets of cooperatively breeding *Neolamprologus pulcher* (Pisces: Cichlidae). — Anim. Behav. 56, 1375-1382.

Taborsky, M. & Limberger, D. (1981). Helpers in fish. — Behav. Ecol. Sociobiol. 8, 143-145.

Taborsky, M. & Taborsky, B. (2015). Evolution of genetic and physiological mechanisms of cooperative behaviour. — Curr. Opin. Behav. Sci. 6, 132-138.

Taves, M.D., Desjardins, J.K., Mishra, S. & Balshine, S. (2009). Androgens and dominance: sex-specific patterns in a highly social fish (*Neolamprologus pulcher*). — Gen. Comp. Endocrinol. 161, 202-207.

Teles, M.C., Gozdowska, M., Kalamarz-Kubiak, H., Kulczykowska, E. & Oliveira, R.F. (2016). Agonistic interactions elicit rapid changes in brain nonapeptide levels in zebrafish. — Horm. Behav. 84, 57-63.

Terranova, J.I., Song, Z., Larkin, T.E., Hardcastle, N., Norvelle, A., Riaz, A. & Albers, H.E. (2016). Serotonin and arginine–vasopressin mediate sex differences in the regulation of dominance and aggression by the social brain. — PNAS 113, 13233-13238.

Thomas, A.X. & Brooks-Kayal, A.R. (2013). Excitation–inhibition epilepsies. — In: Neural Circuit Development and Function in the Brain. Academic Press Inc., Oxford, UK, p. 709-730.

Thompson, R.R., George, K., Walton, J.C., Orr, S.P. & Benson, J. (2006). Sex-specific influences of vasopressin on human social communication. — PNAS 103, 7889-7894.

Thompson, R.R. & Walton, J.C. (2004). Peptide effects on social behavior: effects of vasotocin and isotocin on social approach behavior in male goldfish (*Carassius auratus*). — Behav. Neurosci. 118, 620-626.

Thompson, R.R. & Walton, J.C. (2013). Social regulatory functions of vasotocin and isotocin in fish. — In: Oxytocin, vasopressin and related peptides in the regulation of behavior. Cambridge University Press, Cambridge, UK, p. 75-96.

Tibbetts, E.A., Pardo-Sanchez, J. & Weise, C. (2022). The establishment and maintenance of dominance hierarchies. — Philos. Trans. R. Soc. Lond., B, Biol. Sci. 377, 20200450.

Tinbergen, N. (1953). Social behaviour in animals: with special reference to vertebrates. — Wiley, Oxford, UK.

Torrezani, C.S., Pinho-Neto, C.F., Miyai, C.A., Sanches, F.H.C. & Barreto, R.E. (2013). Structural enrichment reduces aggression in *Tilapia rendalli*. — Mar. Freshwat. Behav. Physiol. 46, 183-190.

Van Dyk, D.A. & Evans, C.S. (2008). Opponent assessment in lizards: examining the effect of aggressive and submissive signals. — Behav. Ecol. 19, 895-901.

Waas, J.R. (1990). Intraspecific variation in social repertoires: evidence from cave-and burrow-dwelling little blue penguins. — Behaviour 115, 63-99.

Wang, F., Flanagan, J., Su, N., Wang, L.-C., Bui, S., Nielson, A., Wu, X., Vo, H.-T., Ma, X.-J. & Luo, Y. (2012). RNAscope: a novel in situ RNA analysis platform for formalin-fixed, paraffin-embedded tissues. — J. Mol. Diagn. 14, 22-29.

Ward, A. & Webster, M. (2016). Sociality: the behaviour of group-living animals. — Springer International Publishing, Cham, Switzerland.

Ward, P.I. & Porter, A.H. (1993). The relative roles of habitat structure and male-male competition in the mating sytem of *Gammarus pulex* (Crustacea; Amphipoda): a simulation study. — Anim. Behav. 45, 119-133.

Wilczynski, W. (1992). Auditory and endocrine inputs to forebrain centers in anuran amphibians. — Ethol. Ecol. Evol. 4, 75-87.

Williamson, C.M., Klein, I.S., Lee, W. & Curley, J.P. (2019). Immediate early gene activation throughout the brain is associated with dynamic changes in social context. — Soc. Neurosci. 14, 253-265.

Wilson, E.O. (2000). Sociobiology: The new synthesis. — Harvard University Press, Cambridge, MA, USA.

Wingfield, J.C. & Sapolsky, R.M. (2003). Reproduction and resistance to stress: when and how. — J. Neuroendocrinol. 15, 711-724.

Wittemyer, G., Douglas-Hamilton, I. & Getz, W.M. (2005). The socioecology of elephants: analysis of the processes creating multitiered social structures. — Anim. Behav. 69, 1357-1371.

Wong, M.Y.L. (2010). Ecological constraints and benefits of philopatry promote group-living in a social but non-cooperatively breeding fish. — Proc. R. Soc. Lond., Ser. B: Biol. Sci. 277, 353–358.

Wong, M.Y.L. & Balshine, S. (2011a). The evolution of cooperative breeding in the African cichlid fish, *Neolamprologus pulcher*. — Biol. Rev. 86, 511-530.

Wong, M.Y.L. & Balshine, S. (2011b). Fight for your breeding right: hierarchy re-establishment predicts aggression in a social queue. — Biol. Lett. 7, 190-193.

Wrona, F.J. & Dixon, R.W.J. (1991). Group size and predation risk: a field analysis of encounter and dilution effects. —Am. Nat. 137, 186-201.

Xi, D., Zhang, X., Lü, H. & Zhang, Z. (2017). Cannibalism in juvenile black rockfish, *Sebastes schlegelii* (Hilgendorf, 1880), reared under controlled conditions. — Aquac. 479, 682-689.

Zayed, A. & Robinson, G.E. (2012). Understanding the relationship between brain gene expression and social behavior: lessons from the honey bee. — Annu. Rev. Genet. 46, 591-615.

Zhang, Z., Zhang, X., Li, Z. & Zhang, X. (2019). Effects of different levels of environmental enrichment on the sheltering behaviors, brain development and cortisol levels of black rockfish *Sebastes schlegelii*. — Appl. Anim. Behav. Sci. 218, 104825.

Zhao, D. & Feng, P. (2015). Temperature increase impacts personality traits in aquatic non-native species: Implications for biological invasion under climate change. — Curr. Zool. 61, 966-971.

Zhou, T., Sandi, C. & Hu, H. (2018). Advances in understanding neural mechanisms of social dominance. — Curr. Opin. Neurobiol. 49, 99-107.

Zöttl, M., Schreier, T. & Taborsky, M. (2023). Coercion promotes alloparental care in cooperative breeders. — Behav. Ecol., arac125.

Zuberbühler, K., Jenny, D. & Bshary, R. (1999). The predator deterrence function of primate alarm calls. — Ethology 105, 477-490.

APPENDIX 1: Submission signals in animal groups

Adam R. Reddon[1,2], Tommaso Ruberto[1] and Simon M. Reader[2]

[1]School of Biological and Environmental Sciences, Liverpool John Moores University, Liverpool, UK

[2]Department of Biology, McGill University, Montreal, Quebec, Canada

This article was published in September 2021 and has been reformatted to fit the thesis requirements.

Published in: Behaviour (Reddon, A. R., Ruberto, T., & Reader, S. M., 2021: Submission signals in animal groups. Behaviour, 159(1), 1-20. https://doi.org/10.1163/1568539X-bja10125)

Abstract

Aggression is costly, and animals have evolved tactics to mitigate these costs. Submission signals are an underappreciated example of such adaptations. Here we review submissive behaviour, highlighting the design of submission signals and how such signals can reduce costs. We focus on non-primates. Animal societies necessitate frequent social interactions, which can increase the probability of conflict. Where maintaining group proximity is essential, animals cannot avoid aggression by fleeing. Mutual interest between group members may also select for efficient conflict avoidance and resolution mechanisms. As a result, submission signals may be especially well developed among group living species, helping social animals to overcome potential costs of recurring conflict that could otherwise counter the benefits of group living. Therefore, submission signalling can be a crucial aspect of social living and is deserving of specific attention within the broader context of social evolution and communication.

Keywords: aggression, agonism, dominance, communication, group living, sociality, submissive

1. Introduction

For many animal species, conflict between conspecifics is unavoidable, because they compete for access to limited resources necessary to survive and reproduce, and this conflict often takes the form of agonistic interactions, (Huntingford et al., 1987; Archer, 1988). There is a rich tradition of studying agonism in ethology and behavioural ecology (Baerends & Baerends-Van Roon, 1950; Lorenz, 1956; Wilson, 1972; Huntingford et al., 1987; Archer, 1988; Hardy & Briffa, 2013). Most of this research effort has been directed towards understanding aggressive behaviour, while submissive behaviour has received far less consideration. For example, in both classic and recent books on animal conflict, little specific consideration is given to submissive behaviour (Huntingford et al., 1987; Hardy &

Briffa, 2013). That said, the neural mechanisms of submission and defence in mammals have received notable attention (reviewed in Adams, 1979; 2006).

Historically, much of the research effort directed at submission has been in the context of the extensive literature on agonism and conflict management in non-human primates (for reviews focused on primates, see Deag, 1977; Bernstein, 1981; de Waal, 1986; Gray & Silverberg, 1992; Sterck et al. 1997; Aureli & de Waal, 2000). However, as others have noted, a focus beyond primates can be informative, and multiple key questions remain unresolved (Silk 2007a; Silk 2007b; Kutsukake & Clutton-Brock, 2008; Seed et al., 2007). Primates also pose a challenge for experimental approaches as significant social manipulations or staging of controlled social interactions is often infeasible. Additional approaches which may provide insights into submission signalling such as experimental evolution or artificial selection studies, developmental manipulations, or pharmacological treatments would also be challenging or impossible in primates but tractable in other taxa.

Here, we highlight submissive behaviour as a broadly underappreciated element of animal social behaviour, with a focus on non-primate social vertebrates. The study of submissive behaviour provides fertile ground to answer questions about conflict resolution, the evolution of communication, signal design, and social information use. Submission signals may be pivotal for group living and thus social evolution, and therefore merit further theoretical and empirical examination. We hope to encourage experimental approaches, as well as observational studies, to investigate submission in a diversity of animals, to help further unravelling the evolution of agonism across social species.

2. Agonism

Aggression is costly, requiring time and energy as well as risking injury (for reviews see: (Huntingford et al., 1987; Hardy & Briffa, 2013). The costs associated with aggression may not differ substantially between the winner and the loser of an interaction (Morrell et al., 2005). For example, aggression-induced stress, energetic costs, and the risk of injury are often similar for both participants (Geist, 1974; Enquist & Leimar, 1990; Brick, 1998; Maan et al., 2001; Earley et al., 2006; Copeland et al., 2011). Both winners and losers also suffer opportunity costs (Grant, 1997), risk attracting predators, and must divert attention away from vigilance (Jakobsson et al., 1995). As a result, contestants often share a mutual interest in minimizing the costs of an interaction (Maynard Smith & Price, 1973; Maynard Smith & Harper, 2003). Because of this mutual interest between competitors, animals are expected to employ less risky forms of aggression than damage inducing attacks (Geist, 1966), such as visual signals (Heathcote et al., 2018), displays (Garamszegi et al., 2006), or vocalizations (Burgdorf et al., 2008). Thus, aggressive behaviour, despite being inherently competitive, often also contains elements of cooperation (Hurd, 1997).

Submissive behaviour includes both avoidance behaviours that allow the focal animal to directly evade aggression, for example fleeing from an aggressor or taking on a protective posture, and submission signals that primarily serve to communicate submission to the receiver (Figure 1). Avoidance behaviours are functionally linked to directly evading aggression but may also have a secondary communicative function, either to signal submission, or as a cue of submission to the aggressing animal. In contrast to avoidance behaviours, submission signals act primarily in communication and become arbitrarily linked to that message, for example a change in body colouration or a vocalisation (i.e., conventional signals; Guilford & Dawkins, 1995). Submission signals are not intrinsically linked to escape, defence, or counterattack, and thus their benefit emerges from its impact on the behaviour of the receiver. The literature on agonism often conflates avoidance behaviour and submission signals, even though the causes and consequences of these different behaviours, and their evolutionary history, may be distinct.

The simplest tactic for an animal to disengage from a conflict is to flee from the aggressor into a new location. However, retreat may not always be a viable option for all species in all contexts (Matsumura & Hayden, 2006). For example, some species may not be sufficiently motile to mount a timely escape (e.g., (Issa & Edwards, 2006; Ligon, 2014).

There may also be ecological limitations on movement, for example in a saturated habitat there may not be another safe location within reach for the loser to flee to (Hatchwell & Komdeur, 2000; Wong, 2010; Batista et al., 2012b). As a result, submission signals are expected to be particularly important when fleeing options are limited. Subordinates of the group living daffodil cichlid fish, *Neolamprologus pulcher*, are more likely to show submission when there are fewer shelters available in the group's territory, therefore decreasing the opportunity to flee from aggression (Reddon et al., 2019). While morphological and ecological factors are the most obvious limitations on escape, social group membership can also be thought of as a constraint on the ability to flee (Wong, 2010; Hick et al., 2014). As we discuss below, the factors favouring group living will limit avoidance behaviours such as fleeing and thus submission signals are expected to be prevalent. In this review, given our emphasis on animal groups, we focus on submission signals rather than avoidance behaviours.

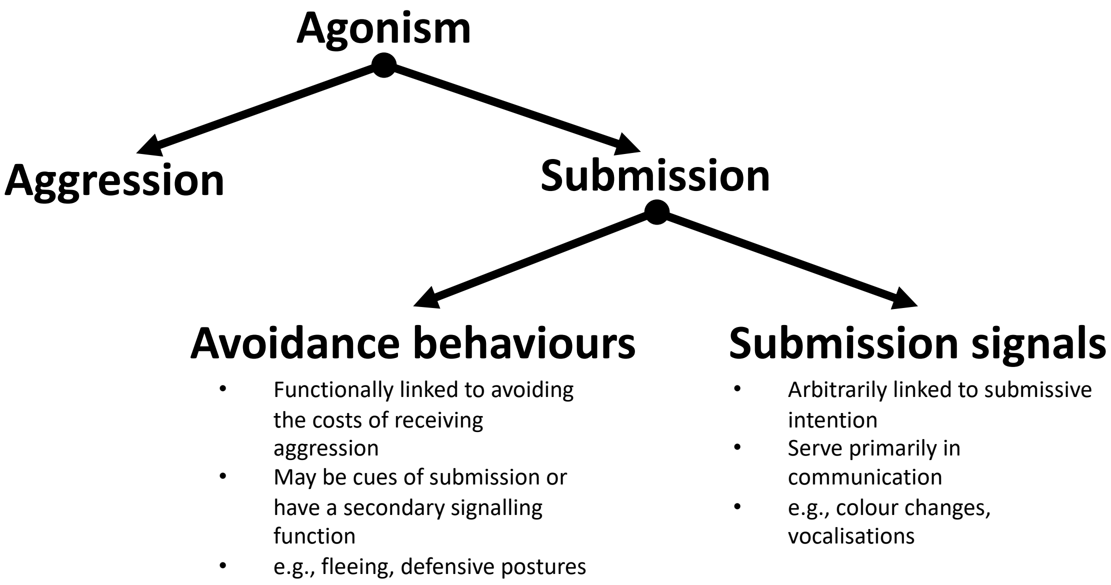

Figure 1. A hierarchical classification of submissive behaviour.

3. Submission signals

Submission signals can be produced prior to any aggressive escalation, pre-empting a contest before it begins, or after fighting begins, to terminate the interaction (Kutsukake & Clutton-Brock, 2006; Kutsukake & Clutton-Brock, 2008). Submission signals benefit the signalling animal by preventing or ending a contest and avoiding further aggression. The receiving animal benefits from accepting a submission signal by reducing the energy and time needed to continue attacking and avoiding the possibility of an upset where the weaker animal unexpectedly prevails. For example, when male crayfish, *Procambarus clarkii*, submit to a competitor by assuming a female-typical mating posture, these pairs show less total aggression, decreased costs of fighting for both individuals, and a reduced chance of death for the signaller than in pairs where the loser does not produce this signal (Issa & Edwards, 2006).

Colour change is a common form of submission signal in exothermic vertebrates. For example, contests between veiled chameleons, *Chamaeleo calyptratus*, end when one individual abruptly darkens their colouration (Ligon, 2014). Darkening colouration leads to a rapid decrease in aggression by the other chameleon, and the likelihood of darkening is tied to the level of aggression received (Ligon, 2014). Similarly, salmonid fishes (Salmo spp.) darken their body and eye colouration, as a signal to their opponent that they relent (Keenleyside & Yamamoto, 1962; O'Connor et al., 1999; Hoglund et al., 2000; O'Connor et al., 2000; Suter & Huntingford, 2002). This darkening inhibits aggression in the receiver, resulting in a precipitous decrease in attack intensity (O'Connor et al., 1999). Much like the chameleons, the amount of aggression that the loser has received in the contest predicts the tendency to darken the body (Ligon, 2014). Other common submission signals include postural changes, for example, in ungulates, turning the antlers away from an opponent (Jennings et al., 2002) or in birds, turning the head to look away from an attacker (Waas, 1990) are used as submission signals. Submission signals need not be visual. For example, in the weakly electric fish, *Gymnotus omarorum*, the losers of a territorial conflict produce electric chirps as a submission signal (Batista et al., 2012b). The latency to produce these chirps decreases while the rate of chirping increases with the intensity of aggression produced by the attacking fish (Batista et al., 2012b).

4. Submission signal design

Submission signals are often highly distinct from aggression signals produced by the same species (Bradbury & Vehrencamp, 2011). Aggression signals may emphasize or exaggerate apparent body size, fighting ability and or motivation to fight, while submission signals tend to minimize these qualities (Huntingford et al., 1987). Signals with incompatible meanings may take on highly distinct forms to make them clearly discriminable by the receiver (Hurd, 1997). As a result, signals that are designed to elicit opposing responses in the receiver tend to evolve towards opposite forms (i.e., the principle of antithesis; (Darwin, 1872). For example, in the red-backed salamander, *Plethodon cinereus*, an amphibian known for frequent and costly fighting, aggressive intent is signalled by an arched back posture, extending the torso high into the air, while submission is signalled by pressing the body down close to the ground (Jaeger, 1984; Dyson et al., 2013). That said, although there are numerous examples of aggression and submission signals that appear to conform to this prediction, to our knowledge, no formal quantitative survey has been done.

Animals often produce several different aggression signals, which indicate increasing willingness to escalate (Hardy & Briffa, 2013). Submission signals could also be similarly graded, with the type, size, or vigour of the signal indicating variation in submissive motivation. In many cases, submission signals are repeated, and distinct forms of submissive behaviour are shown. However, a gesture of limited submission is unlikely to satisfy an aggressor, and therefore unlikely to benefit the signaller (Matsumura & Hayden, 2006). Perhaps as a result, animals typically have a larger repertoire of aggression than submission signals (Bradbury & Vehrencamp, 2011). Moreover, repeated, or diversified submission signals may not indicate strategically graded submission but instead may serve to ensure successful communication. For example, in the brown trout (Salmo trutta), submitting animals darken their body colouration more dramatically in turbid water compared to those in clear water, thereby increasing the strength of the signal, presumably to ensure signal transmission in conditions where visual signals are more difficult to perceive (Eaton & Sloman, 2011).

Some behaviours that have been identified as submission signals may in fact be avoidance behaviours, potentially with a secondary signalling function (Pellis & Pellis, 2015). Moreover, the function of the same action may differ between species and situations (Pellis & Pellis, 2015). For example, in house mice, *Mus domesticus*, rolling on the back does not reduce the likelihood of being bitten and appears to not be a submission signal, unlike some other species, but does help to defend the vulnerable back and rump and facilitates counter-attack and escape (Pellis et al., 1992). Thus, careful analysis is required to identify submission signals, particularly where these signals are derived from pre-existing avoidance behaviour (e.g., through ritualisation; Zahavi, 1980). Complicating matters, submissive behaviours can also act as a ‘hybrid signal’ (Elwood & Prenter, 2013), wherein the same behaviour serves both to provide safety for the sender and to signal submission to the receiver. For instance, wolves, Canis lupus, may lower their ears to signal submission to an aggressor, but this behaviour also helps to protect their vulnerable ears (Beaver, 1999). Like mice, subordinate Norway rats, Rattus norvegicus, roll onto their backs when faced with a dominant aggressor, which in this case does inhibit further aggression from the receiver, but may also provide defensive benefits (Blanchard et al., 1977). Escape or defence behaviours can also act as a cue to the aggressing animal of the submissive motivation of the actor which may affect the aggressor’s subsequent behaviour.

Agonistic interactions are inherently characterized by a conflict of interest between the signaller and the receiver; therefore, agonistic signals require honesty assurances (Bradbury & Vehrencamp, 2011). It is possible to envision a potential benefit from producing a dishonest submission signal in order to lure an opponent into lowering their defences (Dawkins & Guilford, 1997). Honesty in submission signalling could in theory be maintained by production costs (Grafen, 1990), and some submissive behaviours can indeed be energetically costly, for example, subordinate daffodil cichlids increase their routine energy expenditure over three-fold when producing a submissive tail quiver (Grantner & Taborsky, 1998; Taborsky & Grantner, 1998). However, submission signals are often low cost and maintained by mutual advantage to the signaller and receiver (Matsumura & Hayden, 2006). The honesty of submission signals may thus instead be socially enforced (Parker & Rubenstein, 1981; Molles & Vehrencamp, 2001; Webster et al., 2018). The receiver of a dishonest submission signal may punish the signaller by refusing to accept future submission signals from that signaller (Dawkins & Guilford, 1991), or increasing their attack intensity (Clutton-Brock & Parker, 1995; Molles & Vehrencamp, 2001; Van Dyk & Evans, 2008). For example, veiled chameleons, *Chamaeleo calyptratus*, with experimentally manipulated 'dishonest' submissive colouration, inconsistent with their behaviours, received more aggression from dominant individuals than 'honestly' signalling control chameleons (Ligon & McGraw, 2016). Submission signals may place the signalling animal in a vulnerable position or posture (e.g., signaller exposes vulnerable body parts to the receiver; (Lorenz, 1966), making it risky or difficult for the signaller to launch an attack. The resulting positional disadvantage (a vulnerability cost; Adams & Mesterton-Gibbons, 1995) may thereby cancel out any potential benefit of a submissive feint. Submission signals are also typically characterized by a cessation of movement (Pellis & Pellis, 2015), which may reduce the ability for the losing animal to counterattack. Thus, certain postures may have evolved as submission signals for the purpose of enforcing signal honesty. However, as noted above, submission signals might also be at least partly defensive and the apparent vulnerability of submitting animals may have been misinterpreted or overstated in some cases (Pellis & Pellis, 2015). For example, animals lying on their back may be ready to deliver a counterattack rather than exposing vulnerable body areas to their attacker (Schenkel, 1967).

5. Submission signals in social species

Submissive behaviour is likely to play a key role in managing conflict within animal societies (Aureli et al., 2002; Kutsukake & Clutton-Brock, 2006). For obligately social species, leaving one’s current social group can have dire fitness consequences (Heg et al., 2004; Groenewoud et al., 2016). Submission signals have the benefit of preventing or terminating an aggressive interaction without requiring that either participant leave the area or group. In this section, we discuss how submission signals can be of particular benefit to social species by attenuating within-group conflict and thereby facilitating the formation and maintenance of social groups.

Animal groups in which membership is relatively stable, and group members show individual recognition, distinct pairwise relationships, and frequent interactions within the group may appear peaceful but often exhibit some level of intragroup conflict (de Waal, 1986; Aureli et al., 2002; Silk, 2007). The interests of each group member never completely overlap, for example there is often conflict over ranking within the dominance hierarchy and priority access to limited resources (Wong & Balshine, 2011). These conflicts of interest among group members can result in aggressive interactions (Earley & Dugatkin, 2010), which may be costly (Kutsukake & Clutton-Brock, 2008), and if unchecked, may outweigh the benefits of grouping (Aureli et al., 2002). Behaviours that reduce the costs of within-group agonistic interactions represent one of the fundamental building blocks of sociality (Soares et al., 2010; Balshine et al., 2017).

Group living animals also face some additional costs of conflict because of a greater overlap in interests between the interacting parties, compared to animals that do not live in groups (Komdeur & Heg, 2005). Animal groups may be composed of related animals with shared inclusive fitness interests (Hamilton, 1964) which can be negatively affected by intragroup conflict. Groups provide protection and access to resources to their members (Krause & Ruxton, 2002), and these benefits often depend on the size of the group (Kokko et al., 2001(Kokko et al., 2001; Kingma et al., 2014), therefore the loss of productive members through intragroup conflict may negatively affect the remaining membership. In cooperatively breeding species, the success of the group is influenced by the provision of alloparental care which may be lost by inuring, killing, or expelling group members (Kokko et al., 2002). Therefore, even in the absence of relatedness, group success may be an important component of individual fitness, and reductions in the fitness of individual group members may compromise the overall strength of the group (Kokko et al., 2001; Kingma et al., 2014).

Familiarity among social group mates typically results in the formation of a dominance hierarchy based on relative fighting ability (Hand, 1986). Individuals within a dominance hierarchy concede conflicts against group members above them in the hierarchy while prevailing over those of lower rank with minimal aggression (Drews, 1993; Dugatkin & Earley, 2004; Shizuka & McDonald, 2012; Dey et al., 2014; Pini-Fitzsimmons et al., 2021) Shizuka & McDonald 2012; Dey & Quinn 2014; Pini-Fitzsimmons et al., 2021), because familiar animals have likely already observed or experienced each other's fighting abilities (Parker & Rubenstein, 1981; Chase, 1985; Ydenberg et al., 1988; Enquist & Leimar, 1990; Johnsson & Åkerman, 1998). Dominance hierarchies allow the costs of frequent escalated conflicts to be avoided (Senar et al., 1990; Drews, 1993; Pagel & Dawkins, 1997; Bradbury & Vehrencamp, 2011). However, the formation of a dominance hierarchy typically involves a period of increased conflict while the members of the group establish their positions in the social order (Chase et al., 2002; Kura et al., 2015). Unfamiliar animals are initially aggressive with one another but this aggression dissipates as stable dominance relationships form (Drews, 1993). Submission signals may help to facilitate the formation of the dominance hierarchy by reducing the costs of these initial interactions for both parties (Stamps, 1999). Once formed, stable dominance hierarchies are often beneficial for most group members as they result in a net reduction in aggression within the group, and although subordinate members must yield resources to dominants, they can still benefit from belonging to a group (Fischer et al., 2014).

Empirical data support the idea that by signalling submission, a subordinate may reduce their likelihood of being expelled from the group. In social polistine wasps, *Polistes spp*., subordinate individuals remain immobile and hold their head low when challenged by a dominant group member, allowing them to avoid an escalated encounter and possible eviction from the group (Eberhard, 1969). In the daffodil cichlid, more submissive individuals are less likely to be expelled from the group and are permitted to enter a greater number of shelters within the group's territory (Bergmüller et al., 2005; Taborsky et al., 2012). Because of this increased need to maintain a close spatial association with the recipient of submission during intragroup interactions, submission signals are likely to be more commonly used in social species compared to escape behaviours. Ecological constraints on dispersal may be a key driving force underlying the formation of social groups (Hatchwell & Komdeur, 2000), and therefore these effects may reinforce one another. For example, barren habitats may favour group formation as well as limit escape from aggression.

Although aggression is generally lower between familiar group members within an established hierarchy than between unfamiliar individuals, hierarchal societies are not free of agonism (Dey et al., 2013; Reddon et al., 2019). Dominance is often based at least partially on factors that change over time, such as the age and strength of each individual, and as a result, subordinates may occasionally challenge dominants for status (Wong & Balshine, 2011b). Similarly, dominant individuals may benefit from reinforcing their status to discourage future challenges (Buston, 2003; Buston & Cant, 2006; Wong et al., 2007). In either case, subordinate animals may use submission signals to communicate their lack of motivation to challenge for dominance. Therefore, submission signals may play a pivotal role in status-maintaining interactions within established dominance hierarchies. O'Connor at al. (O'Connor et al., 2000) found that familiar subordinate juvenile Atlantic salmon, *Salmo salar*, received less aggression from dominant fish than did unfamiliar fish, but only if they signalled submission, suggesting that the submission signal is an important indication of a lack of aggressive intent from a subordinate to a dominant individual. If submission is shown, then both animals benefit from avoiding further aggression, resulting in a more stable and less costly social relationship (Issa & Edwards, 2006). In this way, submission signals may serve a dual role; being used both in interactions among unfamiliar individuals to conclude acute conflict and between familiar group mates to reinforce dominance relationships. For example, both dogs, *Canis lupus familiaris*, and wolves, *Canis lupus*, use the same submission signals to terminate contests between unfamiliar rivals (Lorenz, 1966) and to maintain the dominance hierarchy within their social group (Schenkel, 1967).

The social complexity hypothesis predicts that animals that live in complex groups require more elaborate communication systems to cope with the greater degree of social intricacy (Freeberg et al., 2012). The hypothesis predicts that animals living in groups have a greater repertoire of social signals composed of a higher number of structurally and functionally distinct elements (Freeberg et al., 2012). It could be argued that the social complexity hypothesis would predict that members of complex animal groups would show a greater diversity of submission signals to cope with the greater variety of potential situations under which submissive behaviour may be elicited and to tailor signals to the variety of different relationships that may exist within a group. However, as discussed above, diversity or gradation in submission signals is not expected, and we believe this prediction will apply to social groups. Therefore, we predict an increased use of submission signals, a broadening of the contexts in which they are produced, and/or a reduction in the threshold required to elicit them within animal groups rather than an increase in submission signal complexity *per se*. Existing data comparing cooperatively breeding birds and fishes to independently breeding ones support this prediction (Hick et al., 2014; Rosa et al., 2016; Balshine et al., 2017). Cooperatively breeding species do not show a greater variety of submission signals compared to their independently breeding relatives, but rather deploy a similar repertoire of submission signals more frequently or in a greater diversity of contexts (Hick et al., 2014). Submission signals likely predate the emergence of complex social living arrangements like cooperative breeding, and evolution may act more on the use of submission signals than their form (Hick et al., 2014).

Because group members interact frequently, there may be an added benefit to maintaining a reputation for signal honesty, thereby incentivizing the use of honest submission signals (Waas, 2006). Social animals also often interact in the presence of other group members, and these eavesdroppers may also gather information about the tendency for particular individuals to use submission signals honestly (McGregor & Peake, 2000; Peake & McGregor, 2004). Thus, dishonest signallers within social groups may face higher social costs in future interactions hence maintaining honest submission signalling within the social group context. Furthermore, where animals can use the behaviour of others to infer social relationships or the salience of social information, this will provide additional routes by which group-living individuals can benefit from submission signalling (Paz-y-Mino et al., 2004; Grosenick et al., 2007; Goossens et al., 2008).

6. Future directions

Some submission signals may be subtle, involving for example, only a minute and transient postural change (Gorlick 1976; Ruberto et al. 2020) or a small alternation to the colouration of a body part (Culbert & Balshine, 2019), and as a result, submission signals may go unnoticed even in well studied systems (Heathcote et al. 2018). This subtle information may provide a route for animals to assess their own or others' competitive ability. We encourage readers to carefully observe agonistic interactions in the animals they study and try to identify submission signals which may have gone heretofore undetected.

Most of the examples of submission signals we have presented are drawn from observational studies. Experiments that manipulate submission and examine the responses to these signals will be essential to critically test predictions. Some possibilities include the use of physical alterations of submission signals, artificial stimuli such as models or computer-generated imagery, and developmental or pharmacological manipulations that alter the expression of submission (e.g., (Roche & Leshner, 1979; Arnold & Taborsky, 2010; Reddon et al., 2012; Taborsky et al., 2012; Hellmann et al., 2015; Taborsky, 2016; Culbert

& Balshine, 2019). Alternatively, the costs and benefits of submission signals could be altered, for example by manipulating the opportunity to flee from a conflict. It may also be possible to experimentally manipulate the information state of the animals by altering the perceived strength of the competitors or the apparent resource value, which may in turn alter the use of submission signals in a systematic way.

Work that compares submissive behaviour in closely related species that differ in their social arrangement or compares within species across populations that vary in their social system (Lott, 1991), will be essential to unravel the coevolution of sociality and submission signals. Further exploration of the role of social context, for example the presence or absence of eavesdroppers, on the expression of submission signals will also help to reveal the interrelationship between social systems and agonistic communication.

7. Conclusions

In this review, we draw attention to submission signals as an important, but understudied, element of the social communication repertoire of animals. We argue that these signals are beneficial for minimizing the costs of conflict and are especially critical when fleeing from an aggressor is costly or impossible. Animals that live in stable social groups benefit from the use of submission signals to establish social order and avoid the potentially prohibitive costs of frequent conflict while remaining in the same group. We hope that this review will inspire investigators to look specifically at the submission signals performed by their study species and strive to understand how these signals influence, and are influenced by, the social and ecological context in which that animal lives.

Acknowledgements

We thank Will Swaney, Sarah Turner, Pierre-Olivier Montiglio, Laura Chouinard-Thuly, Maria Cabrera-Álvarez, and the LOUD POINTS data visualisation discussion group for feedback on previous versions. T.R. was supported by a Liverpool John Moores University Faculty of Science PhD Studentship. A.R.R. was supported by a Banting Postdoctoral Fellowship when this review was originally conceived.

References

Adams, D. B. (1979). Brain mechanisms for offense, defense, and submission – Behav. Brain. Sci. 2, 201–213.

Adams, D. B. (2006). Brain mechanisms of aggressive behavior: An updated review – Neurosci. Biobehav. Rev. 30, 304–318.

Adams, E. S., & Mesterton-Gibbons, M. (1995). The cost of threat displays and the stability of deceptive communication. — J. Theor. Biol. 175, 405-421.

Archer, J. (1988). The behavioural biology of aggression. — Cambridge University Press, Cambridge, UK.

Arnold, C. & Taborsky, B. (2010). Social experience in early ontogeny has lasting effects on social skills in cooperatively breeding cichlids. — Anim. Behav. 79, 621-630.

Aureli, F., Cords, M. & Van Schaik, C.P. (2002). Conflict resolution following aggression in gregarious animals: a predictive framework. — Anim. Behav. 64, 325-343.

Balshine, S., Wong, M.Y.L. & Reddon, A.R. (2017). Social motivation and conflict resolution tactics as potential building blocks of sociality in cichlid fishes. — Behav. Processes 141, 152-160.

Batista, G., Zubizarreta, L., Perrone, R. & Silva, A. (2012). Non-sex-biased dominance in a sexually monomorphic electric fish: Fight structure and submissive electric signalling. — Ethology 118, 398-410.

Beaver, B.V. (1999). Canine behavior: a guide for veterinarians. — WB Saunders Co., Philadelphia, PA, USA.

Bergmüller, R., Heg, D. & Taborsky, M. (2005). Helpers in a cooperatively breeding cichlid stay and pay or disperse and breed, depending on ecological constraints. — Proc. R. Soc. Lond., Ser. B: Biol. Sci. 272, 325-331.

Blanchard, R.J., Blanchard, D.C., Takahashi, T. & Kelley, M.J. (1977). Attack and defensive behaviour in the albino rat. — Anim. Behav. 25, 622-634.

Bradbury, J.W. & Vehrencamp, S.L. (2011). Principles of animal communication (2nd edition). — Sinauer, Sunderland, MA, USA.

Brick, O. (1998). Fighting behaviour, vigilance and predation risk in the cichlid fish *Nannacara anomala*. — Anim. Behav. 56, 309-317.

Burgdorf, J., Kroes, R.A., Moskal, J.R., Pfaus, J.G., Brudzynski, S.M. & Panksepp, J. (2008). Ultrasonic vocalizations of rats (*Rattus norvegicus*) during mating, play, and aggression: Behavioral concomitants, relationship to reward, and self-administration of playback. — J. Comp. Psychol. 122, 357.

Buston, P.M. (2003). Forcible eviction and prevention of recruitment in the clown anemonefish. — Behav. Ecol. 14, 576-582.

Buston, P.M. & Cant, M.A. (2006). A new perspective on size hierarchies in nature: patterns, causes, and consequences. — Oecologia 149, 362-372.

Chase, I.D. (1985). The sequential analysis of aggressive acts during hierarchy formation: an application of the ‘jigsaw puzzle’ approach. — Anim. Behav. 33, 86-100.

Chase, I.D., Tovey, C., Spangler-Martin, D. & Manfredonia, M. (2002). Individual differences versus social dynamics in the formation of animal dominance hierarchies. — PNAS 99, 5744-5749.

Clutton-Brock, T.H. & Parker, G.A. (1995). Punishment in animal societies. — Nature 373, 209-216.

Copeland, D.L., Levay, B., Sivaraman, B., Beebe-Fugloni, C. & Earley, R.L. (2011). Metabolic costs of fighting are driven by contest performance in male convict cichlid fish. — Anim. Behav. 82, 271-280.

Culbert, B.M. & Balshine, S. (2019). Visual threat signals influence social interactions in a cooperatively breeding fish. — Anim. Behav. 151, 177-184.

Darwin, C. (1872). The expression of the emotions in man and animal. — John Murray, London, UK.

Dawkins, M.S. & Guilford, T. (1991). The corruption of honest signalling. — Anim. Behav. 41, 865-873.

Dawkins, M.S. & Guilford, T. (1997). Conspicuousness and diversity in animal signals. — In: Communication. Springer, Boston, MA, USA, p. 55-75.

De Waal, F.B.M. (1986). The integration of dominance and social bonding in primates. — Q. Rev. Biol. 61, 459-479.

Dey, C.J., Dale, J. & Quinn, J.S. (2014). Manipulating the appearance of a badge of status causes changes in true badge expression. — Proc. R. Soc. Lond., Ser. B: Biol. Sci. 281, 20132680.

Dey, C.J., Reddon, A.R., O'connor, C.M. & Balshine, S. (2013). Network structure is related to social conflict in a cooperatively breeding fish. — Anim. Behav. 85, 395-402.

Drews, C. (1993). The concept and definition of dominance in animal behaviour. — Behaviour 125, 283-313.

Dugatkin, L.A. & Earley, R.L. (2004). Individual recognition, dominance hierarchies and winner and loser effects. — Proc. R. Soc. Lond., Ser. B: Biol. Sci. 271, 1537-1540.

Dyson, M.L., Reichert, M.S. & Halliday, T.R. (2013). Contests in amphibians. — In: Animal contests. Cambridge University Press, Cambridge, UK, p. 228-257.

Earley, R.L. & Dugatkin, L.A. (2010). Behavior in groups. — In: Evolutionary Behavioral Ecology. Oxford University Press, Oxford, UK, p. 285-308.

Earley, R.L., Edwards, J.T., Aseem, O., Felton, K., Blumer, L.S., Karom, M. & Grober, M.S. (2006). Social interactions tune aggression and stress responsiveness in a territorial cichlid fish (*Archocentrus nigrofasciatus*). — Physiol. Behav. 88, 353-363.

Eaton, L. & Sloman, K.A. (2011). Subordinate brown trout exaggerate social signalling in turbid conditions. — Anim. Behav. 81, 603-608.

Eberhard, M.J.W. (1969). The social biology of polistine wasps. — Miscellaneous publications (University of Michigan, Museum of Zoology) 140, 1–101.

Elwood, R.W. & Prenter, J. (2013). Aggression in spiders. — In: Animal Contests. Cambridge University Press, Cambridge, UK, p. 113-133.

Enquist, M. & Leimar, O. (1990). The evolution of fatal fighting. — Anim. Behav. 39, 1-9.

Fischer, S., Zöttl, M., Groenewoud, F. & Taborsky, B. (2014). Group-size-dependent punishment of idle subordinates in a cooperative breeder where helpers pay to stay. — Proc. R. Soc. Lond., Ser. B: Biol. Sci. 281, 20140184.

Freeberg, T.M., Dunbar, R.I.M. & Ord, T.J. (2012). Social complexity as a proximate and ultimate factor in communicative complexity. — Philos. Trans. R. Soc. Lond., Ser. B: Biol. Sci. 367, 1785-1801.

Garamszegi, L.Z., Rosivall, B., Hegyi, G., Szöllösi, E., Török, J. & Eens, M. (2006). Determinants of male territorial behavior in a Hungarian collared flycatcher population: plumage traits of residents and challengers. — Behav. Ecol. Sociobiol. 60, 663-671.

Geist, V. (1966). The evolution of horn-like organs. — Behaviour 27, 175-214.

Geist, V. (1974). On the relationship of social evolution and ecology in ungulates. — Am. Zool. 14, 205-220.

Grafen, A. (1990). Biological signals as handicaps. — J. Theor. Biol. 144, 517-546.

Grant, J.W.A. (1997). Territoriality. — Behavioural Ecology of Teleost Fishes, Oxford University Press, Oxford, UK, 81-103.

Grantner, A. & Taborsky, M. (1998). The metabolic rates associated with resting, and with the performance of agonistic, submissive and digging behaviours in the cichlid fish *Neolamprologus pulcher* (Pisces: Cichlidae). — J. Comp. Physiol., B 168, 427-433.

Groenewoud, F., Frommen, J.G., Josi, D., Tanaka, H., Jungwirth, A. & Taborsky, M. (2016). Predation risk drives social complexity in cooperative breeders. — Proc. Natl. Acad. Sci. USA 113, 4104-4109.

Hamilton, W.D. (1964). The genetical evolution of social behaviour. II. — J. Theor. Biol. 7, 17-52.

Hand, J.L. (1986). Resolution of social conflicts: dominance, egalitarianism, spheres of dominance, and game theory. — Q. Rev. Biol. 61, 201-220.

Hardy, I.C.W. & Briffa, M. (2013). Animal contests. — Cambridge University Press, Cambridge, UK.

Hatchwell, B.J. & Komdeur, J. (2000). Ecological constraints, life history traits and the evolution of cooperative breeding. — Anim. Behav. 59, 1079-1086.

Heathcote, R.J.P., Darden, S.K., Troscianko, J., Lawson, M.R.M., Brown, A.M., Laker, P.R., Naisbett-Jones, L.C., Macgregor, H.E.A., Ramnarine, I. & Croft, D.P. (2018). Dynamic eye colour as an honest signal of aggression. — Curr. Biol. 28, R652-R653.

Heg, D., Bachar, Z., Brouwer, L. & Taborsky, M. (2004). Predation risk is an ecological constraint for helper dispersal in a cooperatively breeding cichlid. — Proc. R. Soc. Lond., Ser. B: Biol. Sci. 271, 2367-2374.

Hellmann, J.K., Reddon, A.R., Ligocki, I.Y., O'connor, C.M., Garvy, K.A., Marsh-Rollo, S.E., Hamilton, I.M. & Balshine, S. (2015). Group response to social perturbation: impacts of isotocin and the social landscape. — Anim. Behav. 105, 55-62.

Hick, K., Reddon, A.R., O’connor, C.M. & Balshine, S. (2014). Strategic and tactical fighting decisions in cichlid fishes with divergent social systems. — Behaviour 151, 47-71.

Hoglund, E., Balm, P.H. & Winberg, S. (2000). Skin darkening, a potential social signal in subordinate arctic charr (*Salvelinus alpinus*): the regulatory role of brain monoamines and pro-opiomelanocortin-derived peptides. — J. Exp. Biol. 203, 1711-1721.

Huntingford, F.A., Turner, A.K. & Downie, L.M. (1987). The consequences of animal conflict. — In: Animal Conflict. Springer, Dordrecht, NL, p. 227-250.

Hurd, P.L. (1997). Cooperative signalling between opponents in fish fights. — Anim. Behav. 54, 1309-1315.

Issa, F.A. & Edwards, D.H. (2006). Ritualized submission and the reduction of aggression in an invertebrate. — Curr. Biol. 16, 2217-2221.

Jaeger, R.G. (1984). Agonistic behavior of the red-backed salamander. — Copeia, 309-314.

Jakobsson, S., Brick, O. & Kullberg, C. (1995). Escalated fighting behaviour incurs increased predation risk. — Anim. Behav. 49, 235-239.

Johnsson, J.I. & Åkerman, A. (1998). Watch and learn: preview of the fighting ability of opponents alters contest behaviour in rainbow trout. — Anim. Behav. 56, 771-776.

Keenleyside, M.H.A. & Yamamoto, F.T. (1962). Territorial behaviour of juvenile Atlantic salmon (*Salmo salar* L.). — Behaviour 19, 139-168.

Kingma, S.A., Santema, P., Taborsky, M. & Komdeur, J. (2014). Group augmentation and the evolution of cooperation. — Trends Ecol. Evol. 29, 476-484.

Kokko, H., Johnstone, R.A. & Clutton-Brock, T.H. (2001). The evolution of cooperative breeding through group augmentation. — Proc. R. Soc. Lond., Ser. B: Biol. Sci. 268, 187-196.

Komdeur, J. & Heg, D. (2005). Cooperation and conflict over investment strategies in animals. — Behaviour 142, 1433-1447.

Kura, K., Broom, M. & Kandler, A. (2015). Modelling dominance hierarchies under winner and loser effects. — Bull. Math. Biol. 77, 927-952.

Kutsukake, N. & Clutton-Brock, T.H. (2006). Aggression and submission reflect reproductive conflict between females in cooperatively breeding meerkats *Suricata suricatta*. — Behav. Ecol. Sociobiol. 59, 541-548.

Kutsukake, N. & Clutton-Brock, T.H. (2008). Do meerkats engage in conflict management following aggression? Reconciliation, submission and avoidance. — Anim. Behav. 75, 1441-1453.

Ligon, R.A. (2014). Defeated chameleons darken dynamically during dyadic disputes to decrease danger from dominants. — Behav. Ecol. Sociobiol. 68, 1007-1017.

Ligon, R.A. & Mcgraw, K.J. (2016). Social costs enforce honesty of a dynamic signal of motivation. — Proc. R. Soc. Lond., Ser. B: Biol. Sci. 283, 20161873.

Lorenz, K. (1956). Plays and vacuum activities. — L'instinct Dans le Comportement des Animaux et de l'Homme, Masson, Paris, FR. 633-645.

Lorenz, K. (1966). On aggression. — Routledge, London, UK.

Lott, D.F. (1991). Intraspecific variation in the social systems of wild vertebrates. — Cambridge University Press, Cambridge, UK.

Maan, M.E., Groothuis, T.G.G. & Wittenberg, J. (2001). Escalated fighting despite predictors of conflict outcome: solving the paradox in a South American cichlid fish. — Anim. Behav. 62, 623-634.

Matsumura, S. & Hayden, T.J. (2006). When should signals of submission be given?–A game theory model. — J. Theor. Biol. 240, 425-433.

Maynard Smith, J. & Harper, D. (2003). Animal signals. — Oxford University Press, Oxford, UK.

Maynard Smith, J. & Price, G.R. (1973). The logic of animal conflict. — Nature 246, 15-18.

Mcgregor, P.K. & Peake, T.M. (2000). Communication networks: social environments for receiving and signalling behaviour. — Acta Ethol. 2, 71-81.

Molles, L.E. & Vehrencamp, S.L. (2001). Songbird cheaters pay a retaliation cost: evidence for auditory conventional signals. — Proc. R. Soc. Lond., Ser. B: Biol. Sci. 268, 2013-2019.

Morrell, L.J., Lindström, J. & Ruxton, G.D. (2005). Why are small males aggressive? — Proc. R. Soc. Lond., Ser. B: Biol. Sci. 272, 1235-1241.

O'connor, K.I., Metcalfe, N.B. & Taylor, A.C. (1999). Does darkening signal submission in territorial contests between juvenile Atlantic salmon, *Salmo salar*? — Anim. Behav. 58, 1269-1276.

O'connor, K.I., Metcalfe, N.B. & Taylor, A.C. (2000). The effects of prior residence on behavior and growth rates in juvenile Atlantic salmon (*Salmo salar*). — Behav. Ecol. 11, 13-18.

Pagel, M. & Dawkins, M.S. (1997). Peck orders and group size in laying hens:futures contracts' for non-aggression. — Behav. Processes 40, 13-25.

Parker, G.A. & Rubenstein, D.I. (1981). Role assessment, reserve strategy, and acquisition of information in asymmetric animal conflicts. — Anim. Behav. 29, 221-240.

Peake, T.M. & Mcgregor, P.K. (2004). Information and aggression in fishes. — Anim. Learn. Behav. 32, 114-121.

Pellis, S.M. & Pellis, V.C. (2015). Are agonistic behavior patterns signals or combat tactics—Or does it matter? Targets as organizing principles of fighting. — Physiol. Behav. 146, 73-78.

Pellis, S.M., Pellis, V.C., Manning, J.C. & Dewsbury, D.A. (1992). Supine defense in the intraspecific fighting of male house mice *Mus domesticus*. — Aggressive Behav. 18, 373-379.

Pini-Fitzsimmons, J., Knott, N.A. & Brown, C. (2021). Heterarchy reveals social organization of a smooth stingray (*Bathytoshia brevicaudata*) population in a provisioned food context. — Front. Mar. Sci. 8, 466.

Reddon, A.R., Dey, C.J. & Balshine, S. (2019). Submissive behaviour is mediated by sex, social status, relative body size and shelter availability in a social fish. — Anim. Behav. 155, 131-139.

Reddon, A.R., O'connor, C.M., Marsh-Rollo, S.E. & Balshine, S. (2012). Effects of isotocin on social responses in a cooperatively breeding fish. — Anim. Behav. 84, 753-760.

Roche, K.E. & Leshner, A.I. (1979). ACTH and vasopressin treatments immediately after a defeat increase future submissiveness in male mice. — Science 204, 1343-1344.

Rosa, G.L.M., Ellis, J.M., Bonaccorso, E. & Dos Anjos, L. (2016). Friend or foe? Social system influences the allocation of signals across functional categories in the repertoires of the New World jays. — Behaviour 153, 467-524.

Schenkel, R. (1967). Submission: its features and function in the wolf and dog. — Am. Zool. 7, 319-329.

Senar, J.C., Camerino, M. & Metcalfe, N.B. (1990). Familiarity breeds tolerance: the development of social stability in flocking siskins (*Carduelis spinus*). — Ethology 85, 13-24.

Shizuka, D. & Mcdonald, D.B. (2012). A social network perspective on measurements of dominance hierarchies. — Anim. Behav. 83, 925-934.

Silk, J.B. (2007). Animal behavior: conflict management is for the birds. — Curr. Biol. 17, R50-R51.

Soares, M.C., Bshary, R., Fusani, L., Goymann, W., Hau, M., Hirschenhauser, K. & Oliveira, R.F. (2010). Hormonal mechanisms of cooperative behaviour. — Philos. Trans. R. Soc. Lond., Ser. B: Biol. Sci. 365, 2737-2750.

Stamps, J.A. (1999). *22nd International Ornithological Congress, Durban, South Africa*.

Suter, H.C. & Huntingford, F.A. (2002). Eye colour in juvenile Atlantic salmon: effects of social status, aggression and foraging success. — J. Fish Biol. 61, 606-614.

Taborsky, B. (2016). Opening the black box of developmental experiments: behavioural mechanisms underlying long-term effects of early social experience. — Ethology 122, 267-283.

Taborsky, B., Arnold, C., Junker, J. & Tschopp, A. (2012). The early social environment affects social competence in a cooperative breeder. — Anim. Behav. 83, 1067-1074.

Taborsky, M. & Grantner, A. (1998). Behavioural time–energy budgets of cooperatively breeding *Neolamprologus pulcher* (Pisces: Cichlidae). — Anim. Behav. 56, 1375-1382.

Van Dyk, D.A. & Evans, C.S. (2008). Opponent assessment in lizards: examining the effect of aggressive and submissive signals. — Behav. Ecol. 19, 895-901.

Waas, J.R. (2006). How do Little Blue Penguins “validate” information contained in their agonistic displays? — Adv. Study Behav. 36, 397-447.

Webster, M.S., Ligon, R.A. & Leighton, G.M. (2018). Social costs are an underappreciated force for honest signalling in animal aggregations. — Anim. Behav. 143, 167-176.

Wilson, E.O. (1972). Animal communication. — Sci. Am. 227, 52-63.

Wong, M.Y.L. (2010). Ecological constraints and benefits of philopatry promote group-living in a social but non-cooperatively breeding fish. — Proc. R. Soc. Lond., Ser. B: Biol. Sci. 277, 353–358.

Wong, M.Y.L. & Balshine, S. (2011). Fight for your breeding right: hierarchy re-establishment predicts aggression in a social queue. — Biol. Lett. 7, 190-193.

Wong, M.Y.L., Buston, P.M., Munday, P.L. & Jones, G.P. (2007). The threat of punishment enforces peaceful cooperation and stabilizes queues in a coral-reef fish. — Proc. R. Soc. Lond., Ser. B: Biol. Sci. 274, 1093-1099.

Ydenberg, R.C., Giraldeau, L.A. & Falls, J.B. (1988). Neighbours, strangers, and the asymmetric war of attrition. — Anim. Behav. 36, 343-347.

Zahavi, A. (1980). Ritualization and the evolution of movement signals.— Behaviour, 72, 77-80.

APPENDIX 2: Subordinate submissive responses are predicted by dominant behaviour in a cooperatively breeding fish

Veronica Manara, Tommaso Ruberto, William T. Swaney, Adam R. Reddon

School of Biological and Environmental Sciences, Liverpool John Moores University, Liverpool, UK

This article was published in October 2022 and has been reformatted to fit the thesis requirements.

Published in: Behaviour (Manara, V., Ruberto, T., Swaney, W. T., & Reddon, A. R., 2022: Subordinate submissive responses are predicted by dominant behaviour in a cooperatively breeding fish. Behaviour, 160(2), 127-144. https://doi.org/10.1163/1568539X-bja10192)

Abstract

In complex social groups, animals rely on communication to facilitate priority access to resources and minimise the costs of conflict. Animals typically have more aggression signals than submission signals. However, some social species do show multiple submission signals, and the context in which these different signals are used is often not well understood. In the current study, we assessed agonistic interactions within groups of the cooperatively breeding daffodil cichlid fish (*Neolamprologus pulcher*) to investigate the relationship between the aggressive behaviours of the dominant breeding pair, and the submissive responses of the highest ranked subordinate within the group. Daffodil cichlids may respond to aggression by fleeing or by the production of either a tail quiver display or a head up display. Among the two submission signals, the tail quiver display was used more frequently in response to a threat display while head up displays were produced approximately equally in response to both threat displays and overt aggression. A more exaggerated version of the head up display was given more often in response to overt aggressions, suggesting a graded submissive response both within and between the two submission signals. Within fish, the frequency of head up displays, but not tail quiver displays, correlated positively with the frequency of threat displays received. The current study helps us to better understand the use of graduated submission signals in a highly social vertebrate and sheds light on submission signalling as an understudied aspect of communication.

Keywords: aggression; communication; daffodil cichlid; Neolamprologus pulcher; signalling; submission

1. Introduction

Conflict is costly, and costs may not differ substantially between the winner and loser of an interaction, with energetic expenditure, stress induced physiological responses, and the risk of injury or death, not differing between competitors (Huntingford et al., 1987; Morrell et al., 2005; Hardy & Briffa, 2013). Conflict may also lead to secondary costs, such as reduced vigilance, while increasing the risk of attracting predators (Jakobsson et al., 1995). As a result, animals have evolved strategies to mitigate the costs of conflict (Briffa & Sneddon, 2010). For example, threat displays, opponent assessment, avoidance, and submission may help to minimise costs (Archer, 1988; Hardy & Briffa, 2013; Briffa, 2014). Signals that reliably convey information about aggressive motivation and fighting ability have evolved to benefit both the sender and the receiver of the signal (Smith and Harper, 2003). Aggressive signals usually involve displays that emphasise traits relevant to fighting ability, such as physical strength, size, and weaponry (Huntingford et al., 1987). Conversely, signals that convey submission, usually deemphasise these same traits (Bernstein, 1981; Reddon et al., 2022).

Submission signals are less well characterised in the literature than are aggression or dominance signals, despite being widespread (e.g., (Fox & Cohen, 1977; O'Connor et al., 1999; Ligon, 2014; Ruberto et al., 2020). Most species have fewer submission signals than aggression signals (Bradbury & Vehrencamp, 1998). Although submission signals may be repeated or intensified (Eaton & Sloman, 2011) to ensure the signal is successfully transmitted, multiple distinct submission signals which indicate varying levels of submissive intent are uncommon, as a receiver may be less likely to accept a partial gesture of submission (Matsumura & Hayden, 2006).

Agonistic communication is important for animals that live in groups. Group living is beneficial to the members of the group (Hamilton, 1971; Roberts, 1996; Lehtonen & Jaatinen, 2016). However, living in proximity can increase the likelihood of conflict, which may offset these advantages, and thus conflict within groups must be managed for group living to be a stable strategy (Lorenz, 1966; King, 1973; Krause & Ruxton, 2002). In social groups, the opportunity to flee from an attack may also be reduced by ecological or social constraints (Matsumura & Hayden, 2006; Wong, 2010; Reddon et al., 2022), and therefore social stability relies on effective communication (Frommen, 2020).

The daffodil cichlid (*Neolamprologus pulcher*) is a cooperatively breeding freshwater fish endemic to Lake Tanganyika, East Africa (Taborsky & Limberger, 1981; Balshine et al., 1998). In the wild, daffodil cichlids form stable groups, typically of 3-20 individuals, organised into linear dominance hierarchies based on body size, which correlates with age (Balshine et al., 2001; Dey et al., 2013). The largest male and largest female form a breeding pair who are socially dominant and engage in most of the reproduction in the group. Dominant fish may be replaced due to predation events or territory takeovers, and larger subordinates occasionally disperse between groups (Stiver et al. 2004). Subordinate group members assist the dominant pair in raising their offspring and in defending the territory from predators and competitors (Wong & Balshine, 2011). Within-group agonism is frequent and can result from disputes over status, workload, and resource access (Wong & Balshine, 2011; Fischer et al., 2014; Hick et al., 2014; Balshine et al., 2017). Dominant status is reinforced through threat displays and overt aggression (Dey et al., 2013; Balzarini et al., 2017). Subordinates often flee from dominant aggression by swimming away rapidly (Balshine et al., 2017), however, the ability to flee from aggression may be constrained by the danger of predation outside of the group's territory (Groenewoud et al., 2016) and by the availability of shelters to flee to inside the territory (Reddon et al., 2019). Subordinates may also signal submission through one of two displays: the head up display (HUD; Table 1), or the tail quiver display (TQD; Table 1). Tail quiver displays are given in a variety of social contexts including affiliation and courtship (Pisanski et al., 2015), as well as submission (Bayani et al., 2017; Naef & Taborsky, 2020; Antunes et al., 2022). Head up displays by contrast seem to be used primarily as a submission signal (Ruberto et al., 2020). The HUD may vary in intensity from a slight upwards tilt to the adoption of a near vertical posture in the water column (Sopinka et al., 2009). The cause of this variation in signal expression is unknown, it may be exaggerated to ensure signal transmission in more challenging signalling environments such as the changes in visibility that occur seasonally in Lake Tanganyika, or may represent quantitative variation in submissive motivation, though the latter is not predicted by a model of submission signalling (Matsumura & Hayden, 2006). Head up and tail quiver displays may occur in isolation or together, either sequentially or simultaneously. Previous studies have often focused on one signal or the other (TQD: (Bayani et al., 2017; Naef & Taborsky, 2020; Antunes et al., 2022); HUD: (Reddon et al., 2012; Hick et al., 2014; Ruberto et al., 2020) or combined the two displays into a general submissive display category (Taves et al., 2009). These two different displays may be used in different contexts or may depend on the level of submissive motivation.

In this study, daffodil cichlids belonging to 19 different social groups were observed under standardised environmental conditions. The relationships between aggressive behaviours of the dominant breeding pair, and the submissive responses of the largest subordinate in the group were recorded to examine what predicts the response of the focal subordinate fish. Specifically, how submissive responses varied based on the escalation level of the instigating aggression (threat display or overt aggression). We predicted that the TQD would mostly be used in response to less escalated threat displays, whereas HUDs and fleeing responses would be more frequent in response to overt aggression.

2. Methods

2.1 Study subjects and housing conditions

The research subjects were laboratory reared daffodil cichlids, *Neolamprologus pulcher*, which were descendants (F5-F7) of fish captured on the southern shore of Lake Tanganyika, Africa. Prior to the experiment, all fish were kept in mixed-sex groups of approximately 50 fish per aquarium (105 × 43 x 40cm, 180-litre). The housing tanks were equipped with a heater, a thermometer, two powered filters, an air stone, and 3 cm of fine coral sand. Temperature was maintained at 27 ± 1 C on a 12:12 h light:dark cycle with 15 min of gradual transition in lighting simulating sunrise and sunset. Fish were fed daily with a variety of dried prepared cichlid foods.

The study subjects were later moved into social groups (n = 19) housed in 90L aquaria (53 × 43 x 38 cm), each equipped with two foam filters, a heater, and a thermometer, along with 3 cm of fine coral sand. Each aquarium was furnished with 4 terracotta caves, used by the fish as breeding substrate as well as shelter, and two floating translucent green PET bottles, providing additional refuge. All animals were kept under the same husbandry regime previously described. Fish were housed in either small groups (n = 9), comprised of two dominant individuals and two subordinates, or larger groups (n = 10), which included the two dominant fish and 6-7 subordinates. Sex was only determined in the dominant pair, as many of the subordinates were too small to be sexed by examination of the genital papillae. For each group, the sizes of the dominant breeding pair and of the largest subordinate were recorded, by measuring the standard length of each fish from the tip of the snout to the end of the caudal peduncle (standard length mean ± SD: dominant male = 5.25±0.48cm; dominant female = 4.75±0.52cm; focal subordinate = 3.5±0.45cm). We aimed for approximately a 5-10% difference in body size between breeders, and between adjacent subordinate ranks, with size difference of approximately 25-35% difference between breeder female and largest subordinate, mimicking the size distribution of wild groups. Subordinate individuals were moved into the experimental tanks 24 h before the dominant pair. Groups were housed together for at least one month prior to observation to allow for groups to stabilise while reducing variation across the observation period due to uncertainties in the hierarchy. On the rare occasions that members were rejected during group formation, groups were dissolved, and new ones were formed using new fish from the stock aquaria.

2.2 Video recordings

The 19 groups were recorded with a camera (CX240E Full HD Camcorder, Sony Corp., Japan), from a frontal perspective capturing the entire aquarium. Each group was recorded four times over a period of two weeks between 10:00-15:00, with only one recording captured per day. Each recording was 30 minutes long, leading to a total of 120-minutes of recording per group. The first 10 minutes of each recording were treated as a habituation period and were therefore not coded, resulting in 80 total minutes of coded observation per group, which were summed together for analysis purposes.

2.3 Behavioural coding

We recorded each instance of aggression from either member of the dominant pair towards the largest subordinate (focal fish) and the focal fish's response to that aggression (see Table 1 for a detailed description of the coded behaviours). For each agonistic interaction (n = 369) we recorded the sex of the aggressor and whether they performed an overt aggression or a threat display. The subordinate response was recorded for each interaction as either a submission display (HUD or TQD) or a flee. Moreover, if the submission display was a HUD, the angle of the tilt was visually estimated as being above or below 45° relative to the substrate by pausing the video at the apex of the display. HUD and TQD were recorded as separate responses when they were carried out sequentially or simultaneously in response to a single aggressive act.

Table 1. Ethogram of agonistic behaviour for the daffodil cichlid, Neolamprologus pulcher.

TYPE OF INTERACTION	**BEHAVIOUR**	**DESCRIPTION**
Overt aggression (attacks)	Chase	The dominant fish swims rapidly towards the subordinate for a distance of at least 4 cm.
	Bite/ram	The dominant fish makes contact between their mouth and the body of the subordinate
Threat displays	Aggressive posture	The dominant fish faces the subordinate with their head lowered, tail raised upwards, and fins extended.
	Opercular threat	The dominant fish swims towards the subordinate with its jaws open and opercula extended outward. The pectoral fins are spread,

		while the body is tilted slightly downwards.
Submission (display)	Head up display (HUD)	The subordinate fish responds to an aggression by tilting their body upwards and exposing their ventral aspect to the receiver.
	Tail quiver display (TQD)	The subordinate fish responds to aggression by producing a bilateral oscillation of the tail. Motion originates at the tail and can extend to the entire body.
Submission (escape)	Flee	The subordinate fish responds to aggression by quickly swimming away, for a distance of at least 4 cm.

2.4 Data analysis

To examine the effect of the type of inciting aggression on the type of submissive behaviour elicited, we fit a generalised linear mixed model to a multinomial logistic error structure. Dominant aggression was coded categorically as an overt aggression or a threat display and included as a predictor variable. Although not the focus of this study, we also included the group size (small, large) and the sex of the aggressor (male, female) as predictor variables as the broader social context could affect the submissive responses observed. The behavioural response from the focal fish was coded categorically as a HUD, a TQD, or a flee and included as the response measure. Focal identity was included as a random factor to account for multiple interactions per focal individual contributing to the dataset.

In a follow-up analysis, we compared only the subordinate submission signals (HUD, TQD) depending on the dominant behaviour (overt aggression, threat display), the sex of the dominant and the size of the group, using a generalised linear mixed model fit to a binomial error structure. Group size and dominant sex were included as fixed factors and focal identity was included as a random factor.

The frequencies of HUDs above or below an angle of 45° in response to overt aggression or threat displays were examined with a generalised linear mixed model fit to a binomial error structure. Type of aggressive behaviour received, the size of the group, and the sex of the aggressor were included as predictor variables. The response variable was the angle of the HUD relative to the substrate, coded categorically as HUD >45° or HUD <45°. The identity of the focal fish was included as a random factor. For all models, fixed effects were tested with Wald F tests.

We examined the Pearson product-moment correlation between the number of threat displays or the number of overt aggressions that the focal fish received from the dominant pair with the number of HUDs or TQDs they showed in response ($n = 19$). Data analysis and visualisation were conducted using SPSS (v. 27) and R (v. 3.6.2).

2.5 Ethical statement

Animal housing and handling protocols were approved by the Liverpool John Moores Animal Welfare and Ethics Steering Group (approval number: AR_TR/2018-4) and adhered to the guidelines of the Animal Behaviour Society and the Association for the Study of Animal Behaviour.

3. Results

The type of aggression shown by the dominant fish (overt aggression or threat display) significantly predicted the submissive response of the focal fish ($F_{2,361} = 27.69$, $p < 0.001$, Fig. 1) with flees being much more common in response to overt aggression and TQD being more likely in response to threat displays. Neither group size nor the sex of the aggressor had a significant effect on the focal response (Group size: $F_{2,361}=1.89$, $p = 0.15$; Sex: $F_{2,361}=1.18$, $p = 0.31$).

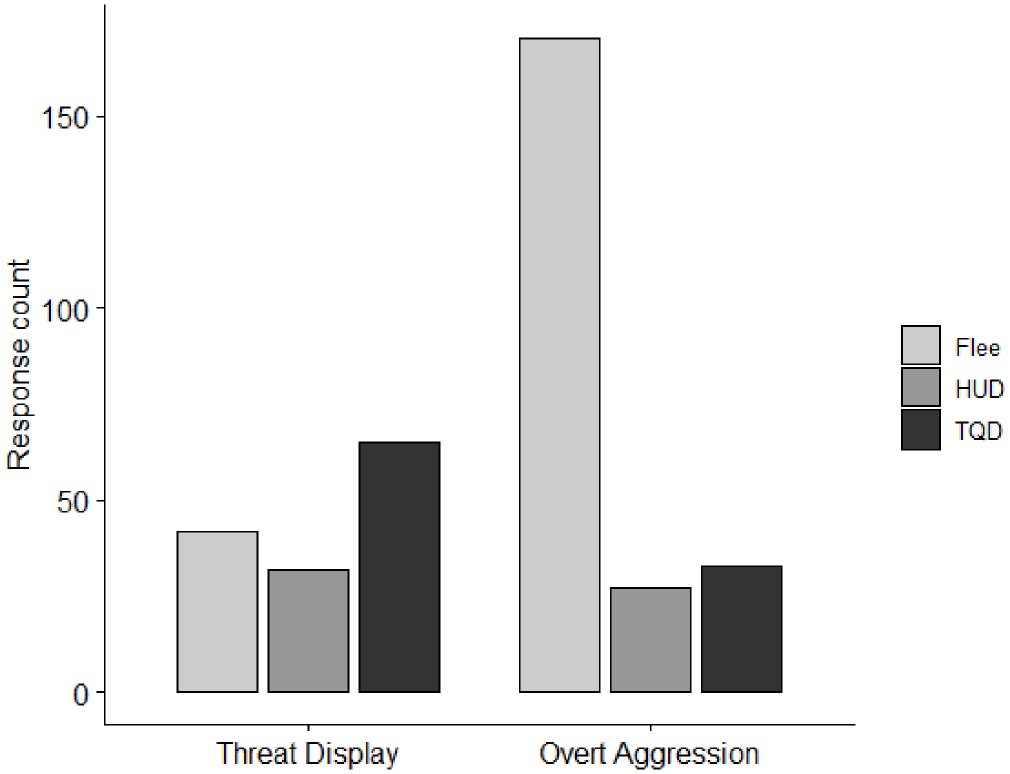

Figure 1. Counts of submissive behaviour in the largest subordinate in response to dominant aggression in 80 minutes of observation. The type of submissive response depended on the type of aggression received ($p < 0.001$).

Restricting the analysis to only submission display responses (i.e., excluding those interactions in which the focal fish fled from the dominant aggression), there was no significant effect of aggression type ($F_{1,153} = 1.49$, p = 0.22), dominant sex ($F_{1,153} = 0.03$, p = 0.86), nor group size ($F_{1,153} = 0.09$, p = 0.76), on the likelihood of the focal fish producing a TQD compared to a HUD in response to dominant aggression.

Aggression type received significantly predicted the likelihood of a HUD being greater than 45° ($F_{1,54}$=5.68, *p*=0.021; Fig. 2), while group size ($F_{1,54}$=0.23, p=0.64) and sex ($F_{1,54}$=0.04, p=0.84) did not.

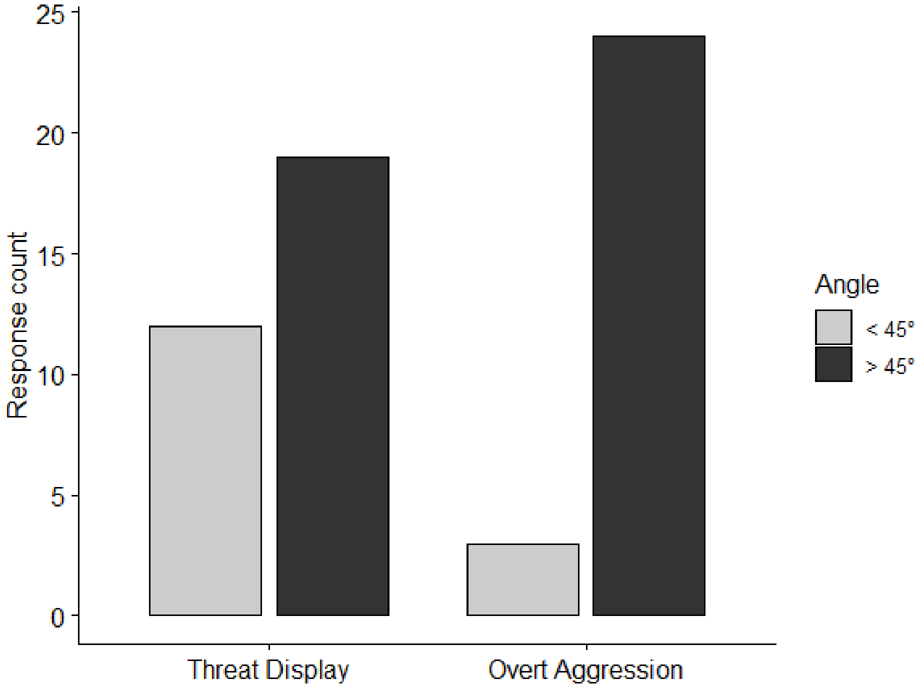

Figure 2. Counts of HUDs elicited above or below 45° relative to the substrate in response to dominant aggression in 80 minutes of observation. The tendency for the HUD to involve a greater tilt of the body was predicted by dominant behaviour (p = 0.02).

There was a significant positive relationship between the number of threat displays from the dominant fish and the number of the HUDs shown by the focal subordinate (r = 0.65, N = 19, p = 0.002, Fig. 3A). In contrast the number of TQDs shown by the focal fish was not significantly predicted by the number of threat displays received (r = 0.27, p = 0.26, N = 19, Fig. 3C). The number of overt aggressions from the dominant pair was not significantly related to the number of HUD shown (r = 0.40, N = 19, p = 0.09, Fig 3B). The number of overt aggressions from the dominant pair was not related to the TQD shown by the focal fish (r = -0.11, N = 19, p = 0.67, Fig. 3D).

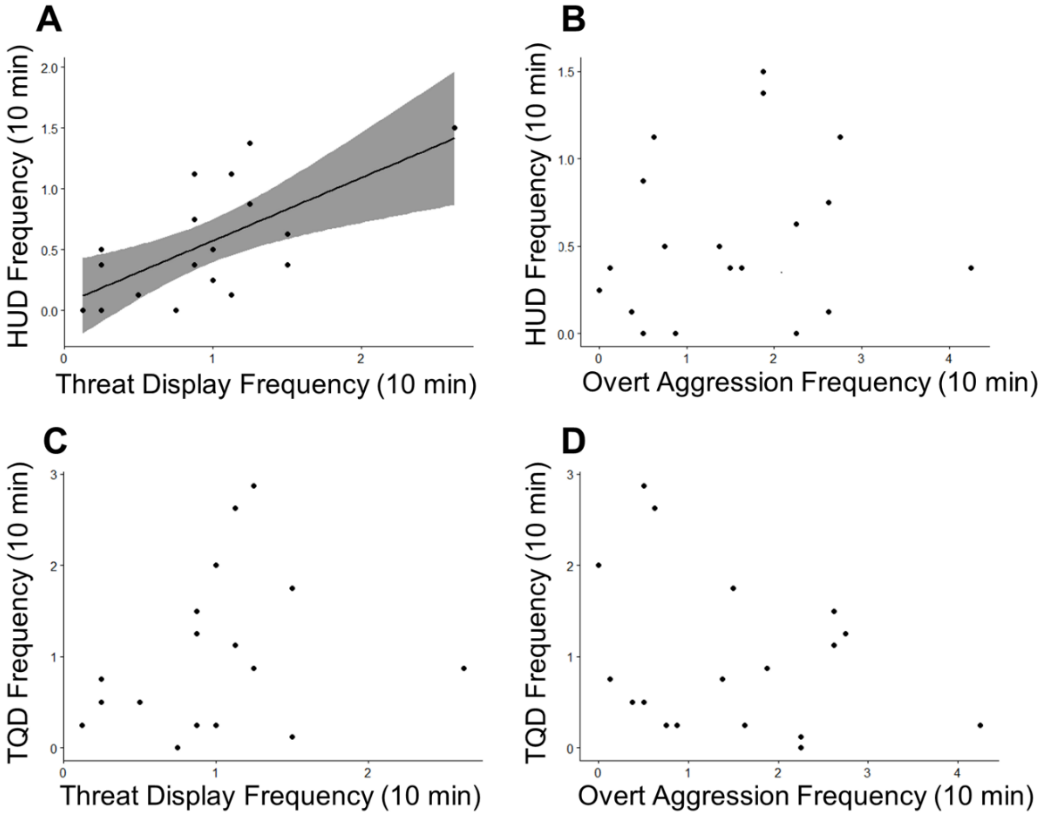

Figure 3. The linear relationship between the aggression received from the dominant fish and the number of submission displays shown by the focal subordinate per 10 minutes of observation. (A) There is a significant positive relationship between the number of threat displays received and the number of HUD shown (p = 0.002). (B) The number of overt aggressions was not significantly related to the number of HUD (p = 0.09). Neither (C) threat displays (p = 0.26) nor (D) overt aggressions (p = 0.67) received from the dominant pair significantly predicted the number of TQD by the focal fish. Linear best fit lines for significant relationships are shown with 95% CI.

4. Discussion

Using detailed observations of 19 laboratory housed groups of daffodil cichlids (*Neolamprologus pulcher*), we found that the escalation level of the aggression shown by the dominant breeding pair towards their largest subordinate helper strongly predicted the resultant submissive response. When one of the dominant pair attacked the focal subordinate by chasing or biting, the subordinate most often fled from the interaction. When the dominant showed a threat display, the focal fish was more likely to show a submission display in response. Both submission signals, head up displays (HUD) and tail quiver displays (TQD), were produced in response to both overt aggression and threat displays from the dominant pair. The number of head up displays was predicted by the number of threat displays received. By contrast, the TQD was not significantly associated with the number of overt aggressions or threat displays received, suggesting the HUD may be a more specialised submission display than the TQD. We also found that the HUD was more likely to be exaggerated through a more dramatic tilt of the body axis when the inciting aggressive act was an overt aggression rather than a threat display, suggesting possible gradation of submissive motivation within signal type.

Both the HUD and the TQD are used as submission signals in the daffodil cichlid, and the context in which they are used overlaps substantially. Submission signals need to be effectively received by dominant individuals to modulate their aggression (Reddon et al., 2022), and daffodil cichlids could use graded or differentiated displays to ensure that signals are efficiently transmitted and received across signalling contexts. For example, brown trout (*Salmo trutta*) show submission by darkening their body colouration, and do so more dramatically in turbid water, possibly to enhance signal transmission when visibility is reduced (Eaton & Sloman, 2011).

Although submissive repertoires are generally not as diverse as aggressive repertoires (Bradbury & Vehrencamp, 1998), examples of animals using more than one submission display are known. For example, Jacky dragons (*Amphibolurus muricatus*) deploy two different submission displays (slow arm waves and slow head bows) during opponent assessment (Carpenter et al., 1970; Van Dyk & Evans, 2008). In canids such as dogs (*Canis l. familiaris*), wolves (*Canis lupus*) and African wild dogs (*Lycaon pictus*), subordinate group members communicate submissive intentions through what is referred to as passive vs. active submission (Schenkel, 1967; Cordoni & Palagi, 2008; Baan et al., 2014; Van den Berghe et al., 2019). These passive and active displays can take place separately or in combination. Both displays involve crouched posture and lowered tail and ears, but these are performed more dramatically in passive displays (Schenkel, 1967). Both displays convey submission, but active displays may also signal affiliative motivation (e.g., during greeting ceremonies), while passive displays are given primarily in response to dominant inquisitive behaviours (e.g., sniffing urogenital areas) or aggression (Schenkel, 1967). Even greater complexity in signal repertoire is observed in social primates such as ring-tailed lemurs (*Lemur catta*). This species' visual and acoustic repertoire includes several submissive postures (Pereira & Kappeler, 1997) and vocalisations (Macedonia, 1993). Interestingly, vocal signals are elicited in context dependent agonistic interactions, with yips, cackles and twitters given by submissive individuals when losing agonistic interactions, and "chutters" elicited by both winners and losers (Bolt, 2021), supporting the existence of intricate signalling systems to mitigate conflict.

As with the canid and primate examples, the relatively rich submissive repertoire of daffodil cichlids could be explained by the social complexity hypothesis for communicative complexity. Socially complex environments can promote the evolution of social cognition, which in turn favours the greater nuance in communication necessary to support behavioural coordination (Freeberg et al., 2012; Sewall, 2015). Cooperative breeding systems are commonly affected by high levels of intra-group social conflict, and the evolution of multiple submission signals in daffodil cichlids could fulfil the need for social context-dependent communication. Alternatively, cooperation per se may not necessarily lead to richer submissive repertoires, but rather may increase the frequency of submission signals and/or reduce the threshold at which they are elicited (Reddon et al., 2022). Recent studies testing these predictions in birds have produced conflicting results (Rosa et al., 2016; Leighton, 2017), highlighting the current lack of agreement surrounding signal evolution in cooperative systems. According to previous analyses comparing cooperative and non-cooperative cichlids, communication repertoires appear to be similarly structured in close relatives across social systems (Hick et al., 2014; Balshine et al., 2017). Furthermore, social complexity should be enhanced when multiple individuals interact, due to eavesdropping and audience effects (Valone, 2007; Zuberbühler, 2008). In the current study however, group size did not affect submission responses, suggesting that eavesdroppers and rank conflict elsewhere in the hierarchy may not have a major effect.

Breeder sex also did not affect the subordinate response, but it should be noted that the sex of the subordinate was unknown, as many individuals were too small to be visually sexed. This lack of information may have obscured sex specific patterns, as aggression from the dominant breeders could depend on whether they are interacting with same versus opposite sex group mates.

The observed overlap between the HUD and TQD may be the result of these signals fulfilling subtly distinct, context-dependent functions. Both signals were elicited in response to dominant aggression, but the HUD was more strongly correlated with threat displays. We recently reported that individuals performing more HUDs receive less aggression from dominant fish (Ruberto et al., 2020). Our current findings seem to indicate that HUDs may be a more specific social signal in daffodil cichlids, serving to de-escalate the dominant aggressive interactions. Moreover, it is possible that differences in the angle of the body tilt in the HUD indicates gradation of submissive motivation in the subordinate fish, allowing the HUD to act as a nuanced submission signal.

The TQD occurred at higher rates in comparison to the HUD but in contrast, it wasn’t significantly correlated with aggression received. This display may be co-opted from other communicative contexts to serve as a submissive-affiliative signal: the TQD is often observed during courtship, and it is conserved across a diverse array of cichlids in this context (Baerends & Baerends-van Roon, 1950; Barlow, 2008), while the HUD appears to be more species-specific. It is possible that subordinate cichlids use the TQD as a multipurpose affiliative-submissive signal, indicating both subordination and affiliation to higher ranked individuals, while the HUD is more strictly used to show immediate submission in response to acute aggression. In other animal species submission signals are reported to serve a multipurpose submissive-affiliative function, as in the case of wolves (Schenkel, 1967; Cafazzo et al., 2010). Displays can be differentiated when used to either de-escalate aggression or to pre-emptively appease the dominant individual, in what are sometimes referred to as submission signals (the former) or subordination (the latter; (Preuschoft & van Schaik, 2000). In wolves, submission signals, such as back rolling, are usually given as an immediate behavioural response following a dominant physical attack, particularly when avoidance behaviours or escape are not a viable option (van Hooff & Wensing, 1987). Subordination signals are primarily directed at dominants outside of the agonistic context, reinforcing subordinate status (van Hooff & Wensing, 1987). Dominant individuals are often approached and greeted by subordinates with stereotypical lowered posture, followed by lip licking and other appeasing behaviours to reemphasise status within an established social relationship. The use of signals for both submission and subordination purposes is also reported in other hierarchical species, such as Verreaux's sifakas (*Propithecus verreauxi;* (Flack & de Waal, 2007)). Chatter vocalisations are used both as immediate response to aggression, but also when aggressive provocation is not apparent, to communicate peaceful intentions, and they are found to reduce within-group conflict while promoting hierarchical stability through reconciliation (Lewis, 2019).

It is possible that the space limitation imposed by the aquarium environment may have affected the conflict management strategies of daffodil cichlids, for example by making fleeing a less viable response, or by increasing the frequency or intensity of aggression from the dominants. Although the behaviour of daffodil cichlids is known to be broadly similar in the wild and in the laboratory (Taborsky & Grantner, 1998), we did find a higher rate of aggression in our observations than has previously been seen in the field (an average of 2 aggressions per 10 minutes in our sample vs. 0.4 per 10 minutes in the field (Hellmann et al., 2015)). Some of this difference may be explained by the difficulty of recording behaviour in the field vs. laboratory setting, with more instances of aggression likely to be missed while observing live via SCUBA compared to coding from high-definition video. It should be noted that space limitations are also present in the wild as this species is highly territorial, defends a relatively small territory (~1m^3), and is exposed to extreme predation threat when venturing outside (Groenewoud et al., 2016). It is possible that subordinates in the wild may make greater use of shelters that are too small to be accessed by the dominants due to the difference in the body size, whereas all shelters were large enough to be used by all fish in our laboratory setup. Future work should examine the importance of shelter size and space limitation on conflict management in daffodil cichlids.

In conclusion, we found that dominant aggressive behaviour was a strong predictor of subordinate submissive responses in the cooperatively breeding daffodil cichlid. The most common response to an overt aggression was to flee, while submission signals were more common in response to a threat display. Both the HUD, and the TQD were used in response to both threat displays and overt aggression. However, the number of HUD shown was predicted by the number of aggressive acts received, while the angle of HUD varied with the type of aggressive behaviour received. These results suggest that the HUD is being used more specifically as a nuanced submission signal, while the TQD may have other social functions. We found evidence to suggest that daffodil cichlid subordinates communicate variation in submissive motivation with both multiple submission signals and variation in the expression of those signals. It is possible that socially complex groups may select for greater nuance in submission signalling than would be expected in one-off interactions.

Acknowledgements

Thanks to Chelsie Hirons-Major for comments on an earlier version of the manuscript. This work was supported by a Royal Society Research Grant (RGSR1191237) to AR. TR was supported by a Liverpool John Moores University Faculty of Science PhD Studentship.

References

Antunes, D.F., Soares, M.C. & Taborsky, M. (2022). Dopamine modulates social behaviour in cooperatively breeding fish. — Mol. Cell. Endocrinol., 111649.

Archer, J. (1988). The behavioural biology of aggression. — Cambridge University Press, Cambridge, UK.

Baan, C., Bergmüller, R., Smith, D.W. & Molnar, B. (2014). Conflict management in free-ranging wolves, *Canis lupus*. — Anim. Behav. 90, 327-334.

Baerends, G.P. & Baerends-Van Roon, J. (1950). An introduction to the study of the ethology of the cichlid fishes. — Brill, Leiden, NL.

Balshine, S., Leach, B., Neat, F., Reid, H., Taborsky, M. & Werner, N. (2001). Correlates of group size in a cooperatively breeding cichlid fish (*Neolamprologus pulcher*). — Behav. Ecol. Sociobiol. 50, 134-140.

Balshine, S., Neat, F.C., Reid, H. & Taborsky, M. (1998). Paying to stay or paying to breed? Field evidence for direct benefits of helping behavior in a cooperatively breeding fish. — Behav. Ecol. 9, 432-438.

Balshine, S., Wong, M.Y.L. & Reddon, A.R. (2017). Social motivation and conflict resolution tactics as potential building blocks of sociality in cichlid fishes. — Behav. Processes 141, 152-160.

Balzarini, V., Taborsky, M., Villa, F. & Frommen, J.G. (2017). Computer animations of color markings reveal the function of visual threat signals in *Neolamprologus pulcher*. — Curr. Zool. 63, 45-54.

Barlow, G. (2008). The cichlid fishes: nature's grand experiment in evolution. — Basic Books, New York City, NY, USA.

Bayani, D.-M., Taborsky, M. & Frommen, J.G. (2017). To pee or not to pee: urine signals mediate aggressive interactions in the cooperatively breeding cichlid *Neolamprologus pulcher*. — Behav. Ecol. Sociobiol. 71, 1-10.

Bernstein, I.S. (1981). Dominance: The baby and the bathwater. — Behav. Brain Sci. 4, 419-429.

Bolt, L.M. (2021). Agonistic vocalization behaviour in the male ring-tailed lemur (*Lemur catta*). — Primates 62, 417-430.

Bradbury, J.W. & Vehrencamp, S.L. (1998). Principles of animal communication. — Sinauer Associates, Inc., Sunderland, MA, USA.

Briffa, M. (2014). Agonistic signals: integrating analysis of functions and mechanisms. — In: Animal signaling and function: an integrative approach. Wiley, Hoboken, NJ, USA, p. 141-173.

Briffa, M. & Sneddon, L.U. (2010). Contest behavior. — In: Evolutionary behavioral ecology. Oxford University Press, Oxford, UK, p. 246e265.

Cafazzo, S., Valsecchi, P., Bonanni, R. & Natoli, E. (2010). Dominance in relation to age, sex, and competitive contexts in a group of free-ranging domestic dogs. — Behav. Ecol. 21, 443-455.

Carpenter, C.C., Badham, J.A. & Kimble, B. (1970). Behavior patterns of three species of Amphibolurus (Agamidae). — Copeia, 497-505.

Cordoni, G. & Palagi, E. (2008). Reconciliation in wolves (*Canis lupus*): new evidence for a comparative perspective. — Ethology 114, 298-308.

Dey, C.J., Reddon, A.R., O'connor, C.M. & Balshine, S. (2013). Network structure is related to social conflict in a cooperatively breeding fish. — Anim. Behav. 85, 395-402.

Eaton, L. & Sloman, K.A. (2011). Subordinate brown trout exaggerate social signalling in turbid conditions. — Anim. Behav. 81, 603-608.

Fischer, S., Zöttl, M., Groenewoud, F. & Taborsky, B. (2014). Group-size-dependent punishment of idle subordinates in a cooperative breeder where helpers pay to stay. — Proc. R. Soc. Lond., Ser. B: Biol. Sci. 281, 20140184.

Flack, J.C. & De Waal, F.B.M. (2007). Context modulates signal meaning in primate communication. — Proc. Natl. Acad. Sci. USA 104, 1581-1586.

Fox, M.W. & Cohen, J.A. (1977). Canid communication. — In: How animals communicate. Indiana University Press, Bloomington, IN, USA, p. 728-748.

Freeberg, T.M., Dunbar, R.I.M. & Ord, T.J. (2012). Social complexity as a proximate and ultimate factor in communicative complexity. — Philos. Trans. R. Soc. Lond., Ser. B: Biol. Sci. 367, 1785-1801.

Frommen, J.G. (2020). Aggressive communication in aquatic environments. — Funct. Ecol. 34, 364-380.

Groenewoud, F., Frommen, J.G., Josi, D., Tanaka, H., Jungwirth, A. & Taborsky, M. (2016). Predation risk drives social complexity in cooperative breeders. — PNAS 113, 4104-4109.

Hamilton, W.D. (1971). Geometry for the selfish herd. — J. Theor. Biol. 31, 295-311.

Hardy, I.C.W. & Briffa, M. (2013). Animal contests. — Cambridge University Press, Cambridge, UK.

Hellmann, J.K., Reddon, A.R., Ligocki, I.Y., O'connor, C.M., Garvy, K.A., Marsh-Rollo, S.E., Hamilton, I.M. & Balshine, S. (2015). Group response to social perturbation: impacts of isotocin and the social landscape. — Anim. Behav. 105, 55-62.

Hick, K., Reddon, A.R., O’connor, C.M. & Balshine, S. (2014). Strategic and tactical fighting decisions in cichlid fishes with divergent social systems. — Behaviour 151, 47-71.

Huntingford, F.A., Turner, A.K. & Downie, L.M. (1987). The consequences of animal conflict. — In: Animal Conflict. Springer, Dordrecht, NL, p. 227-250.

Jakobsson, S., Brick, O. & Kullberg, C. (1995). Escalated fighting behaviour incurs increased predation risk. — Anim. Behav. 49, 235-239.

King, J.A. (1973). The ecology of aggressive behavior. — Annu. Rev. Ecol. Syst. 4, 117-138.

Krause, J. & Ruxton, G.D. (2002). Living in groups. — Oxford University Press, Cary, NC, USA.

Lehtonen, J. & Jaatinen, K. (2016). Safety in numbers: the dilution effect and other drivers of group life in the face of danger. — Behav. Ecol. Sociobiol. 70, 449-458.

Leighton, G.M. (2017). Cooperative breeding influences the number and type of vocalizations in avian lineages. — Philos. Trans. R. Soc. Lond., Ser. B: Biol. Sci. 284, 20171508.

Lewis, R.J. (2019). Subordination signals improve the quality of social relationships in Verreaux's Sifaka: Implications for the evolution of power structures and social complexity. — Am. J. Phys. Anthropol. 169, 599-607.

Ligon, R.A. (2014). Defeated chameleons darken dynamically during dyadic disputes to decrease danger from dominants. — Behav. Ecol. Sociobiol. 68, 1007-1017.

Lorenz, K. (1966). On aggression. — Routledge, London, UK.

Macedonia, J.M. (1993). The vocal repertoire of the ringtailed lemur (*Lemur catta*). — Folia Primatol. 61, 186-217.

Matsumura, S. & Hayden, T.J. (2006). When should signals of submission be given?–A game theory model. — J. Theor. Biol. 240, 425-433.

Morrell, L.J., Lindström, J. & Ruxton, G.D. (2005). Why are small males aggressive? — Proc. R. Soc. Lond., Ser. B: Biol. Sci. 272, 1235-1241.

Naef, J. & Taborsky, M. (2020). Commodity-specific punishment for experimentally induced defection in cooperatively breeding fish. — R. Soc. Open Sci. 7, 191808.

O'connor, K.I., Metcalfe, N.B. & Taylor, A.C. (1999). Does darkening signal submission in territorial contests between juvenile Atlantic salmon, *Salmo salar*? — Anim. Behav. 58, 1269-1276.

Pereira, M.E. & Kappeler, P.M. (1997). Divergent systems of agonistic behaviour in lemurid primates. — Behaviour 134, 225-274.

Pisanski, K., Marsh-Rollo, S.E. & Balshine, S. (2015). Courting and fighting quietly: a lack of acoustic signals in a cooperative Tanganyikan cichlid fish. — Hydrobiologia 748, 87-97.

Preuschoft, S. & Van Schaik, C.P. (2000). Dominance and communication. — In: Natural conflict resolution. University of California Press, Berkeley, CA, USA, p. 77-105.

Reddon, A.R., Dey, C.J. & Balshine, S. (2019). Submissive behaviour is mediated by sex, social status, relative body size and shelter availability in a social fish. — Anim. Behav. 155, 131-139.

Reddon, A.R., O'connor, C.M., Marsh-Rollo, S.E. & Balshine, S. (2012). Effects of isotocin on social responses in a cooperatively breeding fish. — Anim. Behav. 84, 753-760.

Reddon, A.R., Ruberto, T. & Reader, S.M. (2022). Submission signals in animal groups. — Behaviour 1, 1-20.

Roberts, G. (1996). Why individual vigilance declines as group size increases. — Anim. Behav. 51, 1077-1086.

Rosa, G.L.M., Ellis, J.M., Bonaccorso, E. & Dos Anjos, L. (2016). Friend or foe? Social system influences the allocation of signals across functional categories in the repertoires of the New World jays. — Behaviour 153, 467-524.

Ruberto, T., Talbot, J.L. & Reddon, A.R. (2020). Head up displays are a submission signal in the group-living daffodil cichlid. — Behav. Processes 181, 104271.

Schenkel, R. (1967). Submission: its features and function in the wolf and dog. — Am. Zool. 7, 319-329.

Sewall, K.B. (2015). Social complexity as a driver of communication and cognition. — Integr. Comp. Biol. 55, 384-395.

Sopinka, N.M., Fitzpatrick, J.L., Desjardins, J.K., Stiver, K.A., Marsh-Rollo, S.E. & Balshine, S. (2009). Liver size reveals social status in the African cichlid *Neolamprologus pulcher*. — J. Fish Biol. 75, 1-16.

Taborsky, M. & Grantner, A. (1998). Behavioural time–energy budgets of cooperatively breeding *Neolamprologus pulcher* (Pisces: Cichlidae). — Anim. Behav. 56, 1375-1382.

Taborsky, M. & Limberger, D. (1981). Helpers in fish. — Behav. Ecol. Sociobiol. 8, 143-145.

Taves, M.D., Desjardins, J.K., Mishra, S. & Balshine, S. (2009). Androgens and dominance: sex-specific patterns in a highly social fish (*Neolamprologus pulcher*). — Gen. Comp. Endocrinol. 161, 202-207.

Valone, T.J. (2007). From eavesdropping on performance to copying the behavior of others: a review of public information use. — Behav. Ecol. Sociobiol. 62, 1-14.

Van Den Berghe, F., Paris, M.C., Sarnyai, Z., Vlamings, B., Millar, R.P., Ganswindt, A., Cozzi, A., Pageat, P. & Paris, D.B. (2019). Dog appeasing pheromone prevents the androgen surge and may reduce contact dominance and active submission after stressful interventions in African wild dogs (*Lycaon pictus*). — PLoS ONE 14, e0212551.

Van Dyk, D.A. & Evans, C.S. (2008). Opponent assessment in lizards: examining the effect of aggressive and submissive signals. — Behav. Ecol. 19, 895-901.

Van Hooff, J.A. & Wensing, J.A. (1987). Dominance and its behavioral measures in a captive wolf pack. — In: Man and wolf: Advances, issues, and problems in captive wolf research. Dr. W. Junk Publishers, Dordrecht, NL, p. 219.

Wong, M.Y.L. (2010). Ecological constraints and benefits of philopatry promote group-living in a social but non-cooperatively breeding fish. — Proc. R. Soc. Lond., Ser. B: Biol. Sci. 277, 353–358.

Wong, M.Y.L. & Balshine, S. (2011). The evolution of cooperative breeding in the African cichlid fish, *Neolamprologus pulcher*. — Biol. Rev. 86, 511-530.

Zuberbühler, K. (2008). Audience effects. — Curr. Biol. 18, R189-R190.